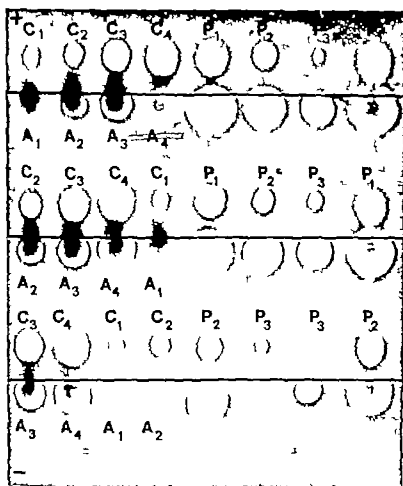


Fig. 6 Inhibition zones of ampicillin and cloxacillin after separation by high voltage electrophoresis. The patient was 7 days' old girl. Samples taken after 1 (P_1) 3 (P) and 5 (P_3) hours after one administration of streptomycin 6.5 *mg i.m., ampicillin 37 mg i.* and *cloxacillin 29 mg i.* per kg b.w. The black horizontal lines represent the place of application. Each sample is applied on 4 different points, (the last application of P on bottom line has failed). The inhibition zones of ampicillin are under the lines and cloxacillin above. No inhibition zones of streptomycin are visible. Standard series cloxacillin C_1 8 C 16, C_3 32 and C 64 µg/ml ampicillin A_1 2 A_2 4 A_3 8 and A : 16 µg/ml. + anode, − cathode.

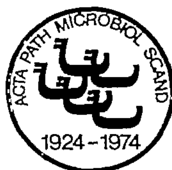
This problem was overcome by digestion of the plasma proteins before the electrophoretic separation of the antibiotics. The protein digestion results in an array of cloxacillin independent of the presence of albumin. The implication of these observations in the discussion of protein binding of antibiotics is evident, but has not yet been an object of further studies. From a practical point of view the total amount of antibiotic is recorded by both methods.

The statistical analysis of the electrophoretic method shows a confidence interval that does not differ very much from that of the disc diffusion method. Both methods require two standard series if the confidence interval is to be improved. *Lightbown & de Rosa* (8) found a confidence limit of ± 4 per cent by analysing tetracycline and oleandomycin in combination.

Certain disadvantages are involved in the present technique. The procedure requires a

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TABLE 2. *Percentage Distribution, by Species of 212 Yeasts Isolated from the Various Specimen Groups*

Specimen group	*Candida albicans* %	*Candida parapsilosis* %	*Candida tropicalis* %	*Candida krusei* %	*Candida guilliermondii* %	*Torulopsis glabrata* %	*Trichosporon cutaneum* %	*Pityrosporum orbiculare or ovale* %	*Rhodotorula rub a* %	*Saccharomyces cerevisiae* %
	53.2	19 4			1.6	3.2	4.8	1..9	4.8	
sing sputum l cavity	78.1		9 4	3.1		6.3				3 1
pharynx	52.9		11.8			33.3				
ina	33.6		11 1	11 1		11 1		11 1		
es	60 0	3.0	3.0			22.5				7.5
ne	30 7	5.8	3.8			48.1		11.5		
al	52.8	8.0	4 7	0.9	0.5	21.2	1 4	7 1	1 4	1.9

TABLE 3. *Total Growth of Y asts in Fungal Cultures of Skin Specimens and most Common Yeast Species by Location*

pecimen group	Cultivated specimens, total No.	Specimens, positive for yeasts %	Isolated yeast strains, total No.	*Candida albicans* %	*Candida parapsilosis* %	*Torulopsis glabrata* %	*Pityrosporum orbiculare or ovale* %	*Trichosporon cutaneum* %
t	32	40 4	24	20.8	37.5	4.2	20.8	8.3
dinal fold	6	66.7	5	60 0			20 0	20.0
er limb	7	28.6	4	75.0		25.0		
t	11	81.8	10	40.0	20.0		20.0	
mmary d area	11	55.6	7	85 7	14.2			
s, varia	43	27.9	12	100.0				
al	130	41.5	62	53.2	19 4	3.2	12.9	4.8

was unexpectedly low as compared with the present author s earlier results and those col lected by *Gentles & La Touche* (1969) and with the incidence of 47 6 per cent found by *Marples* (1966) in subjects over 60 years of age The greater part of the patients in the present series were toothless, part of them using dentures every effort was made to observe good oral hygiene.

Candida albicans grew from 25 4 per cent of the specimens from the skin, which can be held to be a reasonable incidence since skin lesions were common in these patients. Half of the specimens from actual skin folds were positive for this yeast (Table 3) as could be expected (*Lodin* 1975) Healthy skin and faultless urinary tracts usually yield *Candida albicans* in less than 1 per cent (*Seebacher et al.* 1971 *Schönebeck & Ansèhn* 1972) In the present study this yeast was isolated from urine in 14.5 per cent, faeces in 27.5 per cent and vaginal discharge in 31.5 per cent of specimens. The majority of patients from whom the urine samples were taken had chronic urinary tract infection. Many of the strains isolated from urine were undoubtedly contaminations from the vagina and the gut about one third of the patients had inconti-

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THE MOUSE MUTANT *NUDE* DOES NOT DEVELOP SPONTANEOUS TUMOURS

An Argument Against Immunological Surveillance

Jørgen Rygaard and C. O. Povlsen

The Pathological Anatomical Institute, Kommunehospitalet, DK 1399 Copenhagen K, Denmark

The mouse mutant *nude* is immunologically deficient due to congenital absence of the thymus. It will accept allografts and heterografts from closely and remotely related donors. Its humoral immune response to a number of other antigens is also imperfect. *De novo* malignancies can be induced in the *nude* by chemical carcinogen. The lack of a cell-mediated immune response could be expected to be accompanied by a similar defect in the immunological surveillance mechanism against neoplasia. We have studied 11,000 *nude* mice from birth to three months of age or to the normal life expectation of the animal. The total observation time within the period 1969-1973 was some 40,000 months of mouse life. No malignancy was observed during this period. This striking observation is discussed in relation to *de novo* cancers following organ transplantation in man, and the high incidence of malignancies in patients with immune deficiency disorders. It is suggested that immunological surveillance—if existing—may represent a third expression of the immune system, separate from cell-mediated and humoral immunity.

It is supposed that the immune system protects the organism not only from exogenous aggression, infection and foreign tissues for example but also from endogenous aggression most importantly malignancy. This latter theory was proposed by *Thomas* (1959) and has received wide support. *Burnet* (1970a) described the process as "immunological surveillance"— the concept that a major function of the immunological mechanisms in mammals is to recognize and eliminate foreign patterns arising in the body by somatic mutation or by some equivalent process (*Burnet* 1970b).

Prehn & *Lappé* (1971) opined that "anyone with the temerity to question "the theory's overriding importance is likely to be the subject of discrete but possibly welldeserved ridicule" and indeed the weight of support to the hypothesis is considerable, in particular that lent by the studies of patients under immuno-suppressive therapy (*Penn* 1970, *Penn et al.* 1971, *Starzl et al.* 1971, *Penn* & *Starzl* 1972) and patients with immune deficiency disorders (*Good* 1970). Studies in neonatally thymectomized animals are also significant (*Defendi* & *Roosa* 1964, *Miller et al.* 1964, *Law* 1966, *Allison* & *Taylor* 1967, *Gaugas et al.* 1969). Comprehensive reviews have been made by *Burnet* (1970b), *Smith* & *Landy* (1970) and others e.g. *Penn* (1970), *Möller* (1971).

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ULTRASTRUCTURAL LOCALIZATION OF BLOOD GROUP ANTIGEN A AND CELL COAT ON HUMAN BUCCAL EPITHELIAL CELLS

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Departments of Oral Pathology Royal Dental Colleges Copenhagen and Århus, Department of Electron Microscopy Royal Dental College, Århus, Denmark, and Laboratoire de Médecine expérimentale, Collège de France Paris, France

The ultrastructural localization of blood group substance A and its relationship to the cell coat was studied on exfoliated buccal epithelial cells from four male subjects. The antigens were detected by a double layer immunoperoxidase staining method (IP) as well as a double layer immunoferritin (IF) staining method. The cell coat was studied using ruthenium red. There was no variation in results obtained by the IP and IF staining procedures. Positive reacting cells (IP and IF staining) showed either an even or a patchy distribution of the reaction products along the outer part of the cell membrane. Some cells had only very little or no reaction products. The cell coat appeared as an evenly distributed electron dense granular layer at a localization corresponding to that of the antigen.

Previous studies have shown that the blood group antigens A and B occur not only in the erythrocytes but also have a widespread distribution throughout the tissues (11 27). In epithelia the antigens have been found to be associated with the cell surfaces of the stratified and transitional types and with the secretion products of the mucous glands (9 12, 27). In the oral mucosa blood group antigens have been demonstrated along cell membranes in the spinous cell layer of the epithelial surface and in the mucous-secreting glands (1 25, 26). This has been shown by the use of the mixed cell agglutination technique (25) immunofluorescence (1 5, 26) and immunoperoxidase staining techniques (5) all techniques used at the light microscopical level.

Electron microscopy with ferritin or peroxidase-conjugated antibodies has been used frequently to localize specific antigens at the surface of various cell types (2, 10 13 15 18 23). According to our knowledge, however studies describing the distribution of blood group antigen in oral epithelium at the ultrastructural level are not available.

Therefore the purpose of the present work has been to investigate the ultrastructural localization of blood group antigen A and its relationship to the cell coat on normal human buccal epithelial cells.

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Requests for reprints should be addressed to E. Dabelsteen Department of Oral Pathology Royal Dental College, 4 Universitetsparken, DK 00 Copenhagen Ø Denmark.

MATERIAL AND METHODS

Cell Preparation

Exfoliated squamous epithelial cells from the buccal mucosa were obtained from four healthy male

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NEUTROPHIL GRANULOCYTE FUNCTION *IN VITRO*

Evaluation of a fluid phase leucocyte-bacteria reaction system

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A modified method for evaluation of human neutrophil granulocyte function in vitro based upon the combined determination of total and intracellular surviving bacteria in a reaction system of leucocytes and *Staphylococcus aureus* is described. Analysis of the system reveals a close relationship between the processes of ingestion and intraleucocytic killing on a functional level, and points towards dependence of intraleucocytic killing rate upon the rate of ingestion. Defects in intraleucocytic killing are disclosed readily by an increase in the number of surviving intracellular bacteria which will not be caused by increased ingestion under normal conditions. Greatly impaired ingestion will cause an increase in total surviving bacteria with a near normal number of intracellular surviving bacteria. On the basis of these studies it can be concluded that this type of method is particularly suitable for the detection of defects in intraleucocytic killing whereas defects in ingestion are less readily disclosed.

Evaluation of the phagocytic function of neutrophilic granulocytes has received increased attention within later years and a number of congenital and acquired defects associated with decreased resistance to bacterial and fungal infections are being reported. These disease syndromes can be related to serum factors (2, 3, 4, 18, 25) or to the cells, and among the latter to isolated or combined defects in attachment and ingestion (6, 19, 24) or intraleucocytic killing of bacteria and fungi (1, 7, 16, 23).

In vitro incubation of leucocytes with bacteria in the presence of autologous serum followed by colony counting of surviving bacteria will detect abnormalities related to serum factors, ingestion, and intraleucocytic killing but is particularly suitable for detection of defective opsonization (15). The effect of variations in serum factors can be excluded by using a standard serum but distinction between ingestion and intraleucocytic killing remains a major technical problem. In such a system the final indicator is the number of viable bacteria, or colony-forming units (CFU), and it is therefore clear that any recorded reduction in initially present CFU is dependent upon the combined rates of ingestion and killing. Effective elimination of non-ingested bacteria with antibiotics (8, 21) and blocking of further intraleucocytic killing subsequent to reaction of the cells with the bacteria (21) have however been major improvements towards a more precise evaluation of the individual processes of ingestion and intraleucocytic killing.

This study describes a modification of the in vitro leucocyte function test of *Alexander*

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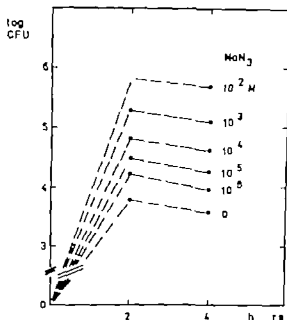


Fig. 2 Effect of increasing molarity of sodium azide, as indicated in the figure, upon the intracellular recoverable colony-forming units (CFU) *Staph. aureus* from a reaction system of normal leucocytes and *Staph. aureus*
Mean of two experiments.

groups of cells is shown in Figure 4 There is a marked increase in intracellular CFU when normal cells are treated with azide (circles) A slight increase in intracellular CFU was seen from untreated to azide-treated C.G.D heterozygote cells (triangles) This is surprising since C.G.D carriers are supposed to harbour a normal and a defective cell population (24) and azide would render all the cells defective. Even if cells from the homozygote C.G.D patients were used azide seems to increase to a minor extent the number of recoverable intracellular CFU (squares) In Figure 5 the effect of 10^{-4} M sodium azide is compared with that of phenylbutazone, 2 mg per ml —another known inhibitor of intraleucocytic killing (21,23) If total CFU were recorded, complete inhibition of killing by normal cells seems to be achieved with both agents, but if intracellular CFU were recorded, the blocking effect of azide seems to be slightly superior to that of phenylbutazone. Since, however the effect of azide was slightly decreased in the simultaneous presence of phenylbutazone this might partly be explained by an inhibitory effect of phenylbutazone upon the ingestion, whereby fewer bacteria would be taken up in the cells, and therefore more bacteria killed by the antibiotics in the surrounding medium Phenylbutazone thus inhibits both ingestion and intraleucocytic killing of *E coli* by guinea pig polymorphonuclear leucocytes (23)

To study whether azide was bound to the cells normal leucocytes were incubated for 30 minutes at 35 C with rotation in the presence and absence of 2 1 × 10^{-4} M sodium azide. The cells were then washed thrice at low-speed centrifugation followed by reaction with the test-organism in the usual manner The results shown in Figure 6 indicate that azide exerts no permanent effect upon resting cells. This suggests that azide either diffuses freely across the cell mem-

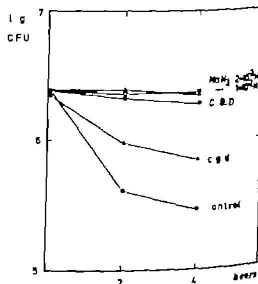


Fig 5 Total recoverable colony-forming units (CFU) *Staph. aureus* from reaction systems of *Staph. aureus* and 1) normal leucocytes (● ten experiments in four persons) 2) leucocytes from patients with chronic granulomatous disease (C.G.D) (■ eight experiments in five patients) 3) leucocytes from female carriers of C.G.D. (c.g.d.) (▲ eight experiments in seven carriers) and 4) normal leucocytes in the presence of sodium azide (NaN) 2 × 10^{-3} M (□; four experiments in three persons) and 1 × 10^{-3} M (△ two experiments in two persons)

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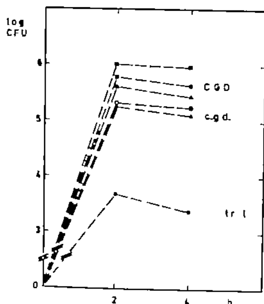


Fig. 4 Intracellular recoverable colony-forming units (CFU) *Staph. aureus* from reaction systems of *Staph. aureus* and 1) normal leucocytes (● six experiments in four persons) 2) leucocytes from patients with chronic granulomatous disease (C.G.D.) (■ four experiments in three patients) and 3) leucocytes from female carriers of C.G.D. (c.g.d.) (▲ three experiments in three carriers) Open symbols represent intracellular recoverable CFU from the corresponding leucocytes in the presence of sodium azide (NaN) 1 × 10 M (same number of experiments)

brane, but is not firmly bound to its site of action, when the cells are not engaged in phagocytosis, or that the membrane of resting cells is not permeable to azide

DISCUSSION

Intracellular killing of a number of microorganisms, including *Staph. aureus* which is katalase positive, is probably largely mediated through the action of hydrogen peroxide, generated through the increased oxydative metabolism subsequent to ingestion, in conjunction with myeloperoxidase of the leucocytes and possibly an oxydizable cofactor (11 18, 19) Sodium azide inhibits the antibacterial activity of an isolated system made up of these components and that of the intact cells (12) The effect of azide can be ascribed to inhibition of the enzymatic activity of the myeloperoxidase of the cells. Azide will also inhibit the iodination of bacteria which can be brought about by H_2O_2 in the presence of peroxidase and iodide (11)

In the present study killing of *Staph. aureus* by normal leucocytes was completely inhibited by 10^{-4} M azide if the total recoverable CFU was used as the indicator (Figure 1) By measuring recoverable intracellular CFU the inhibition was shown to be even further increased from 10^{-4} to 10^{-3} M azide since even more CFU could be recovered intracellularly from cells treated with 10^{-} than cells treated with 10^{-4} M azide (Figure 2) Two possibilities could explain this finding One is that the recording of recoverable intracellular CFU is a more sensitive method of demonstrating decreased intraleucocytic killing which at least in part can be explained by the elimination of con-

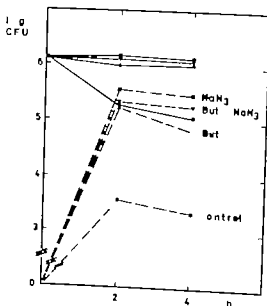


Fig 5 Total recoverable colony-forming units (CFU) *Staph. aureus* (———) and intracellular recoverable CFU (-----) from reaction systems of *St ph. au eus* and 1) normal leucocytes (●) 2) normal leucocytes in the presence of phenylbutazone (But) 2 mg per ml (▲) 3) normal leucocytes in the presence of sodium azide (NaN_3) 1 × 10^{-3} M (■) and 4) normal leucocytes in the presence of But and NaN in the same concentrations (▼)

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Angiopathy essential hypertension, delayed hypersensitivity
Antibodies, smooth muscle, indirect immunofluorescence
Antibody hepatitis B, hepatitis B antigen, radioimmunoassay *Staphylococcus aureus*
Antigen, cell membrane, oral epithelium, blood group antigen, ultrastructure
Antigen, hepatitis B, hepatitis B antibody radioimmunoassay *Staphylococcus aureus*
Antigenic structure, *Staphylococcus aureus*, phage pattern, horse strains
Antigens, carcinoembryonic, nonspecific cross-reacting antigens, alpha protein, immunodiffusion
Antigens, kidney human, characterization, kidney hetero antibodies, rabbit, lymphocytotoxicity
Antigens, nonspecific cross-reacting, carcinoembryonic antigens, alpha protein, immunodiffusion
Antistaphylolysin, detection, plate-diffusion
Antistreptolysin O reversed single radial immunodiffusion

TABLE 1 *Human Antibodies to Toxoplasma Comparison of Titres Obtained with Three Serological Tests*

Serum number	Indirect immuno-peroxidase test	Methylene blue dye test	Indirect hemag glutination test
1	8192	8192	2048
	8192	8192	4096
3	8192	8192	512
4	8192	4096	512
5	4096	4096	2048
6	4096	4096	512
7	4096	2048	2048
8	2048	2048	4096
9	2048	2048	2048
10	2048	1024	1024
11	1024	1024	4096
12	1024	512	4096
13	1024	512	16
14	512	512	1024
15	256	512	1024
16	256	256	512
17	256	128	128
18	128	128	512
19	128	32	512
20	64	64	32
21	64	16	32
22	32	32	32
23	32	32	16
24	32	16	32
25	32	16	32
26	16	32	512
27	16	16	256
28	16	8	32
29	8	8	32
30	8	<4	16
31	4	<4	16

sda in a solution of 5 mg 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Mo) in 10 ml of 0 05 M Tris buffer pH 7.6 and 0.1 ml 1 per cent H_2O_2 which was added just before using. This was followed by two 15 min rinses in cold PBS and a 2 min rinse in distilled water air-drying and then mounting in DPX (DePex, Gurr Ltd., London, England) The mounting step was considered optional unless slides were to be stored for reference. Control slides to which PBS was added instead of serum were included. It should be noted that thorough washing with agitation between steps in this procedure is very important. The slides were observed through a Leitz light microscope at a magnification of × 320 or × 800 using a blue clear filter (Beck and Sohne Kassel, Germany)

Human Sera

Sera were obtained from the diagnostic serology laboratory They were selected according to their DT titres; high, medium and low titres (Table 1)

Additional Toxoplasma Serological Tests

Before performing the IIPT antibody titres to *Toxoplasma* were determined by the methylene blue dye test (DT) (7) and the indirect hemag glutination test (IHAT) (15) The same dilution preparations were used in all three tests (Table 1)

RESULTS

The final endpoint titration was from a positive brown to a negative gray or gray-white colored parasite (Fig. 2 and 3) In low dilutions, the periphery of the organism tended to be a darker brown than the center giving the appearance of a dark ring around the parasite. With higher dilutions, the brown color tended to be more evenly distributed over the entire parasite, since the peripheral color was not as pronounced It was found that approximately 5 per cent of parasites in a positive titration might be gray-colored Polar staining, as seen in the indirect fluorescent antibody test (IFAT) (24) was not apparent with the IIPT

Negative reactions were obtained with control slides incubated with only conjugate, normal human serum or PBS Control slides to which only peroxidase in high concentration (0.5 mg/ml) was applied showed very minimal activity

Slides to which only 3,3'-diaminobenzidine H_2O was applied were included to check for endogenous peroxidase activity by the parasites(1) Only minimal endogenous peroxidase activity could be detected, and this could be inhibited by initially treating the parasite slides for 15 or 30 min at room temp in a solution of 10 ml absolute methanol containing 0.02 ml concentrated (37 per cent) HCl (23)

However it was found that by not pretreating parasites with methanol-HCl the brown color of red blood cells, which have endogenous peroxidase activity could be compared in the same field with the degree

Arbovirus, *Ixodes ricinus* passerine birds 297
Arboviruses, Semliki Forest virus, mutation, viral RNA synthesis 810
Arthritis, rheumatoid, rheumatoid factors, cross-reaction, IgG 733
Arthrobacter mutants, protease formation 657
Ascaris suum faecal egg counts, worm burdens 879
Ascaris suum migrating larvae, eosinophil response, congenitally thymus-less mice 919
Aspergillus fumigatus indirect haemagglutination, antibodies 871
Australia-SH antigen, immunodiffusion, complement fixation test, electron microscopy 151
Bacillus cereus phospholipase C, screening method 354
Bacteria, respiratory tract, agar culture, "Pitting" and "Corrosion" 48
Bacteriophage, *Mycobacterium renae (fortuitum)* typing 87
Bacteriuria, luminol chemiluminescence, urinary tract infection 575
Bile acid sulphates, metabolization, bacteria 537
Blood bactericidal activity treponema immobilization test, syphilis serodiagnosis 185
Blood group antigen, oral epithelium, ultrastructure, cell membrane antigen 113
Borrelia merionesi Borrelia recurrentis flagella, cell division 799
Borrelia recurrentis Borrelia merionesi flagella, cell division 799
Cancer spontaneous, immune deficiency in mice, thymus, tumour immunology 99
Candida albicans blood monocytes, phagocytosis, *in vitro* 235
Candida albicans radiolabelling, electrolytes 219
Candida albicans Torulopsis glabrata opportunistic infections 81
Carcinomas, renal cellular hypersensitivity serum inhibiting activity leucocyte migration 294
Casein, amyloidosis, mouse spleen cells, cellular immunity 287
C3 convertase properdin, complement, crossed immunoelectrophoresis 914
Chromatography mycotoxin, *Stachybotrys alternans* tissue culture toxicity 7
Complement, first component, esterases, purification 590
Complement fixation test, immunodiffusion, electron microscopy Australia-SH antigen 151
Complement, immunoelectrophoresis 585
Complement, properdin, C3 convertase crossed immunoelectrophoresis 914
Cowdria ruminantium, multiplication tick cell culture 455
Culture tick cell, *Cowdria ruminantium* multiplication 455
Cystic fibrosis, *Pseudomonas aeruginosa* B and T lymphocytes, immunoelectrophoresis
Cystic fibrosis, *Pseudomonas aeruginosa* immune response, immunoelectrophoresis
Cystic fibrosis, *Pseudomonas aeruginosa Staphylococcus aureus* epidemiology
Cytotoxicity transformation, radiotherapy lymphoma
Dehydrase, 6-phosphogluconate 6-phosphogluconate dehydrogenase 2-keto-3-deoxy-6-phosphogluconate aldolase, *Neisseria*
Dehydrogenase, glucose 6-phosphate, glucokinase, electrophoresis, *Neisseria*
Dehydrogenase glutamate, malate dehydrogenase *Neisseria immunological comparison*
Dehydrogenase malate, glutamate dehydrogenase, *Neisseria* immunological comparison
Dehydrogenase, 6-phosphogluconate 6-phosphogluconate dehydrase, 2-keto-3-deoxy-6-phosphogluconate aldolase, *Neisseria*
Dental plaque, endocarditis, *Streptococcus mutans* properties
Ecthyma contagiosum, epidemiology electron microscopy
Egg counts, faecal, *Ascaris suum* worm burdens
Electrophoresis, paper microbiological assay penicillins, drug combinations
Electrophoresis, polyacrylamide-gel immunofluorescence, haemagglutination, T mycoplasmas
Endocarditis, dental plaque, *Streptococcus mutans*, properties
Enzymes, bacteria, small intestines, slaughter pigs
Eosinophil response, *migrating larvae, Ascaris suum* congenitally thymus-less mice
Erysipelothrix coccoides altered immune response, plaque assay
Epithelium, oral blood group antigen, ultrastructure cell membrane antigen
Escherichia coli L-phase variants, L-colony production
Escherichia coli Mycoplasma arthritidis pyelonephritis
Escherichia coli opsonins, neutrophils, glucocorticoids
Escherichia coli resistance factors, elimination, anthelmintics
Fatty acids, *Micrococcaceae* gas chromatography taxonomy
Fatty acids, monosaccharides, bacteria, gas chromatography

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Fatty acids, *Neisseraceae* gas chromatography taxonomy 767
Flagella, *Borrelia merionesi* *Borrelia recurrentis* cell division 799
Fluorescent antibody technique, diagnosis, I-ruses, respiratory tract infection 41
Fluorescent antibody technique, *Herpes simplex* diagnosis, staphylococcus, cell wall protein A 323
Fusiformis nucr phorus polysaccharide accumulation 635
Galactose neuraminic acids, interferon, leucocytes 505
Gas chromatography bacteria, fatty acids, monosaccharides 753
Gas chromatography fatty acids, *Micrococcus* taxonomy 785
Gas chromatography fatty acids, *Neisseraceae* taxonomy 767
Gel-diffusion tests, leprosy antibodies 701
Glucocorticoids, opsonins, neutrophils, *Escherichia coli* 696
Glucokinase, glucose 6-phosphate dehydrogenase, electrophoresis, *Neisseria* 201
Graft-versus-host reaction, thymus, cortisol, amyloidosis 719
Graft-versus-host reactions, bone marrow transplantation, T cell precursors, cell fractionation 724
Granulocytes, neutrophil, phagocytosis, phenylbutazone 258
Haemagglutination, immunofluorescence, polyacrylamide-gel electrophoresis, T mycoplasmas 345
Haemagglutination, indirect, antibodies, *Aspergillus fumigatus* 871
Haemolysins and enzymes, streptococcal groups A, C, G isoelectric focusing 860
Haemophilus phr philus, septicaemia, bacteriological diagnosis 745
Haemophilus classification, porphyrins 835
Haemophilus influenzae serotyping, immunoelectrophoresis 164
Haptens, immune tolerance, antibody specificity immunologic memory 567
Herpes simplex diagnosis, fluorescent antibody technique, staphylococcus, cell wall protein A 323
Heterogeneity electrophoretic, antibodies, *Klebsiella pneumoniae* rabbits 429
Hyaluronidase activ ty aminopeptidase activity *Str ptococ us mitis* 521
Hyaluronidase, aminopeptidase, *Streptococcus mitis* extraction 593
Hyaluronidase, aminopeptidase, *St eptococcus mitis* growing cells 608
Hyaluronidase aminopeptidase, *St ptococcus mitis* non-growing cells 615
Hyaluronidase repression, *St ptococcus mitis* 625
Hypergammaglobulinaemia, *N sem cuniculi*, transplacental transmission, blue fox
Hypersensitivity cellular leucocyte migration, serum inhibiting activity renal carcinomas
Hypersensitivity delayed, essential hypertension, angiopathy
Hypersensitivity recurrent aphthous stomatitis, humoral immunity immunofluorescence
Hypertension, essential angiopathy delayed hypersensitivity
IgE, nephropathies
Immune deficiency in mice, thymus, spontaneous cancer tumour immunology
Immunity cellular mouse spleen cells, amyloidosis, casein
Immunity cellular d mouse amyloidosis, spleen graft
Immunity tumour SV40-tumour diffusion chambers
Immunodiffusion, carcinoembryonic antigens, nonspecific cross-reacting antigens, alpha protein
Immunodiffusion, complement fixation test, Australia-SH antigen, electron microscopy
Immunodiffusion, reversed single radial, antistreptolysin O
Immunoelectrophoresis, complement
Immunoelectrophoresis, crossed, properdin, C3 convertase, complement
Immunoelectrophoresis, cystic fibrosis, B and T lymphocytes, *Pseudomonas aeruginosa*
Immunoelectrophoresis, cystic fibrosis, immune response, *Pseudomonas aeruginosa*
Immunoelectrophoresis, *Haemophilus influenzae* serotyping
Immunofluorescence, haemagglutination, polyacrylamide-gel electrophoresis, T-mycoplasmas
Immunofluorescence, indirect, rubellavirus, IgM-antibodies, IgA-antibodies
Immunofluorescence, indirect, smooth muscle antibodies
Immunofluorescence, recurrent phthous stomatitis, hypersensitivity humoral immunity
Immunological competence impaired, nosematosis, mice
Immunoperoxidase test, antibodies, *Toxoplasma gondii*
Infection, urinary tract, bacteriuria, luminal chemiluminescence
Infection, urinary tract, *Mycoplasma primatum* isolation
Infection, *Yersinia enterocolitica* *Yersinia pseudotuberculosis* antibodies

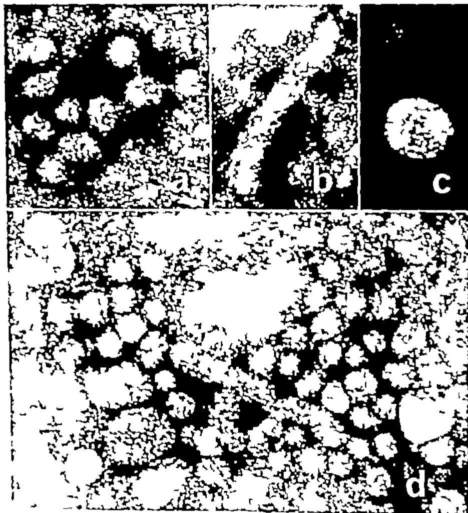


Fig. 1 Electron micrographs showing the structures usually found to be associated with the Australia-SH antigen. Small globular particles aggregated by addition of antiserum specific to the Australia-SH antigen. *b* Tubular elements. Large globular particle with visible substructure. *d* Different Australia-SH antigen associated particles aggregated by specific antiserum. (Magnification 360,000)

diluted antiserum and complement (guinea pig serum, four units of complement) were mixed. The complement fixation took place at 4° C overnight. After addition of 0.025 ml sensitized sheep red cells, the samples were incubated at 37° C for 40 min. The red cells were allowed to settle for two hours at 20° C before the results were read. No others but serum samples which reacted with both antisera were recorded as containing the Australia-SH antigen.

Four of the serum samples were not tested because of scarcity of serum.

The complement fixation tests were performed by persons who did not know the results obtained by the other methods.

Other laboratory tests. Standard procedures used at The Department of Infectious Diseases, Oslo City Hospital, Oslo, were used to determine the amount of bilirubin, glutamic oxalo-acetic transaminase (SGOT), glutamic pyruvic transaminase (SGPT) and alkaline phosphatase in the serum samples from the patients.

EXPERIMENTS AND RESULTS

The results are summarized in Tables 1 and 2. The first serum sample obtained from the four patients from whom serial samples were

Infections, viral, vaccine protection, interferon, interferon production 904
Inhibiting activity serum, renal carcinomas, cellular hypersensitivity leucocyte migration 294
Inhibition, cell migration, leprosy 707
Inhibition, leucocyte migration, non-immunological inhibition 277
Inhibition, non-immunological, leucocyte migration inhibition 277
Interferon, interferon production, vaccine protection, viral infection 904
Interferon, leukocytes, neuraminic acids, galactose 305
Intestines, small, bacteria, enzymes, slaughter pigs 644
Isoelectric focusing haemolysins and enzymes, streptococcal groups A, C, G 860
Ixodes ricinus arbovirus, passerine birds 297
Klebsiella pneumoniae antibodies, electrophoretic heterogeneity rabbits 429
Lactobacillus casei folic acid, antibiotics 371
Larvae, migrating *Ascaris suum* eosinophil response, congenitally thymus-less-mice 919
Leprosy cell migration inhibition 707
Leprosy mycobacterial infections, antibodies, gel-diffusion tests 701
Leukocytes, interferon, neuraminic acids, galactose 305
Luminol chemiluminescence, bacteriuria, urinary tract infection 375
Lymphocyte traffic, post-capillary venules, electron microscopy *nude* mice 249
Lymphocyte transformation, phytohaemagglutinin, sarcoidosis 122
Lymphocytes, B and T cystic fibrosis, *Pseudomonas aeruginosa*, immunoelectrophoresis 559
phocytotoxicity kidney antigens, human, characterization, kidney hetero antibodies, rabbit 409
Lymphoma, radiotherapy cytotoxicity transformation 263
Lysolecithin, bacteria, L-forms, viruses, protozoa, yeast 748
Memory immunologic immune tolerance, haptens, antibody specificity 567
Microbiological assay paper electrophoresis, penicillins, drug combinations 67
Micrococcaceae fatty acids, gas chromatography taxonomy 785
Microgametogony schizogony *T xoplasma gondii* ultrastructure 167
Migration, leucocyte cellular hypersensitivity serum inhibiting acti ity renal carcinomas 294
Mitomycin, C, DNA, tissue culture 270
Monocytes, blood, functional properties, structural properties, *in itro* 223
Monocytes, blood, phagocytosis, *Cand d albicans in vitro*
Monosaccharides, fatty acids, bacteria, gas chromatography
Moraxella, Acinetobacter thymidine phosphorylase, nucleoside deoxyribosyltransferase, thymidine kinase
Moraxella osloensis infections, streptomycin resistance, penicillin resistance
Mycobacteria DNA, isolation, purification
Mycobacterial infections, cell migration inhibition, leprosy
Mycobacterial infections, leprosy antibodies, gel-diffusion tests
Mycobacterium bovis M tuberculosis infection, *Arvicola terrestris*
Mycobacterium leprae leprosy cell migration inhibition
Mycobacterium ranae (fortuitum) bacteriophage, typing
Mycobacterium tuberculosis M bovis infection, *Arvicola terrestris*
Mycoplasma arthritidis pyelonephritis, *Escherichia coli*
Mycoplasma primatum urinary tract infection isolation
Mycoplasmas, canine, classification
Mycoplasmosis, experimental pyelonephritis, antibody
Mycoplasmosis, *Mycoplasma arthritidis* pyelonephritis, *Escherichia coli*
Mycotoxin, *Stachybotrys alternans*
Mycotoxin, *Stachybotrys alternans*, chromatography tissue culture toxicity
Mycotoxins, *Stachybotrys alternans* biological assays
Mycotoxins, *Stachybotrys alternans* pregnancy complications, mice
Neisseria enzymes, intermediary metabolism
Neisseria glucokinase, glucose 6-phosphate dehydrogenase electrophoresis
Neisseria glutamate dehydrogenase, malate dehydrogenase, immunological comparison
Neisseria meningitidis competence variants, osmotic pressure, survival and turbidity changes
Neisseria meningitidis purine metabolism, adenine utilization
Neisseria meningitidis purine metabolism, utilization, adenosine, guanosine inosine
Neisseria 6-phosphogluconate dehydrogenase 6-phosphogluconat dehydrase 2-keto-3-deoxy-6-phosphogluconate aldolase
Neisseria thymidine phosphorylase nucleoside deoxyribosyltransferase thymidine kinase
Nephropathies, IgE

tests were found to contain the antigen. Among the serum samples obtained during the course of serum hepatitis, the number of Australia-SH antigen-containing samples found by electron microscopy was about the same as that found by the complement fixation test. Thus, with a view to revealing this antigen, the former test is at least as sensitive as the complement fixation test, and more sensitive than the agar gel double diffusion test, although the sensitivity of the latter test could be increased by increasing the amount of the serum sample to be tested.

These results are in agreement with those obtained by *Cossart et al.* (1971). Likewise, *Drouhet et al.* (1972) found electron microscopy to be somewhat more sensitive than the agar gel double diffusion test. In a study of different dilutions of Australia-SH antigen-containing sera, *Shulman & Barker* (1969) found an increasing sensitivity of the three test systems in the following order: agar gel double diffusion, electron microscopy and complement fixation.

However the electron microscopical test seems to be less specific than previously presumed. Structures indistinguishable from the Australia-SH antigen associated particles have been observed by electron microscopy in serum of an apparently healthy student (*Solaas* 1973). In the present study small amounts (less than four observations per sample) of presumed Australia-SH antigen could be identified using this test as the only method, in 33 per cent of the patients from a point source epidemic of hepatitis, and in 50 per cent (four out of eight) of the healthy blood donors.

At face value this could imply that small quantities of Australia-SH antigen are present in a considerable number of healthy persons. In agreement with this, *Apostolov et al.* (1971) found by counter electrophoresis and complement fixation, but not by agar gel double diffusion, a presence of Australia-SH antigen in urine concentrates obtained from two out of seven healthy persons. The antigen was not demonstrable in their sera. Furthermore it has been calculated that only about 25 per cent of serum hepatitis cases may be prevented by exclusion of blood donors whose sera react with antiserum to Australia-SH antigen, as determined by agar gel double diffusion tests (*Gocke et al.* 1970).

On the other hand, if sera where three or less observations were made contain structures which are not true Australia-SH antigen particles, this diagnostic procedure failed in four out of 14 serum samples (28.6 per cent) which by agar gel double diffusion were shown to possess the Australia-SH antigen and in seven out of 18 (38.9 per cent) samples where the antigen was detected by the complement fixation test.

In the present study addition of antiserum specific to the Australia-SH antigen prior to electron microscopical examination failed to aggregate the particles observed in seven serum samples. This suggests that some of the structures observed are not true Australia-SH antigen particles, unless the immunological test system was greatly out of balance (*Solaas* 1973). Whether the particles observed in the healthy blood donors were agglutinated by the antiserum could not be evaluated. The antigen in three samples was observed only if no antiserum had been added and in one sample only after addition of antiserum.

In eight samples aggregated presumed Australia-SH antigen associated particles were observed by electron microscopy prior to addition of antiserum. None of these sera showed anticomplementary activity. Furthermore, antibody to the Australia-SH antigen could not be observed in any of these samples, either by agar gel double diffusion or by complement fixation tests (unpublished results). The significance of a presence of such aggregates in serum samples is not known.

The use of an antiserum specific to the Australia-SH antigen and a presence of proper controls ensure the specificity of the agar gel double diffusion test.

Antisera obtained from multiply transfused patients may contain more than one antibody specificity and antigen-antibody reactions other than the specific Australia-SH reaction

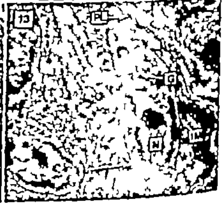
Neuraminic acids, galactose interferon, leukocytes 305
Neutrophil granulocytes, phagocytosis 127
Neutrophil granulocytes, phagocytosis, functional defect 439
Neutrophil granulocytes, phagocytosis, sodium azide 136
Neutrophils, opsonins, glucocorticoids, *Escherichia coli* 696
Nosema cuniculi transplacental transmission, blue fox, hypergammaglobulinaemia 299
Nosematosis, impaired immunological competence, mice 451
Nucleocapsid, viral, Semliki Forest virus, virus replication, messenger ribonucleoprotein 33
Nucleoside deoxyribosyltransferase, thymidine phosphorylase, thymidine kinase *Moraxella Acinetobacter* 57
Nucleoside deoxyribosyltransferase, thymidine phosphorylase, thymidine kinase, *Neisseria* 53
Nude mice, post-capillary venules, lymphocyte traffic, electron microscopy 249
Nude mouse, amyloidosis, cellular immunity spleen graft 403
Ochratoxin A, natural occurrence, cereals 301
Oncogenic potential, decreased, SV40-transformed cells, diffusion chambers, peritoneal cavity 687
Opportunistic infections, *Candida albicans Torulopsis glabrat* 81
Opsonins, neutrophils, glucocorticoids, *Escherichia coli* 696
Penicillin resistance streptomycin resistance, *Moraxella osloensis* 518
Penicillins, drug combinations, microbiological assay paper electrophoresis 67
Phage pattern, *Staphylococcus aureus*, antigenic structure, horse strains 899
Phagocytosis, blood monocytes, *Candida albicans in vitro* 235
Phagocytosis, neutrophil granulocytes 127
Phagocytosis, neutrophil granulocytes, functional defect 439
Phagocytosis, neutrophil granulocytes, phenylbutazone 258
Phagocytosis, neutrophil granulocytes, sodium azide 136
Phenylbutazone, phagocytosis, neutrophil granulocytes 258
Phospholipase C, *Bacillus cereus* screening method 354
Phylogeny staphylococcal protein A-reactive globulins, birds, mammals 12
Phytohaemagglutinin, lymphocyte transformation, sarcoidosis 122
"Pitting" and "Corrosion agar culture bacteria, respiratory tract 48
Plaque assay altered immune response, *Eperythrozoon coccoides*
Polysaccharide accumulation *Fusiformis necrophorus*
Polysaccharides, cell wall, staphylococci, animal
Porphyrins, *Haemophilus*, classification
Precursors, T cell, bone marrow transplantation, graft-versus-host reactions, cell fractionation
Properdin complement, C3 convertase, crossed immunoelectrophoresis
Proteus inconstans (providencia) urea positive strains
Pseudomonas aeruginosa cystic fibrosis B and T lymphocytes immunoelectrophoresis
Pseudomonas aeruginosa cystic fibrosis, immune response, immunoelectrophoresis
Pseudomonas aeruginosa Staphylococcus aureus cystic fibrosis, epidemiology
Purine metabolism, *Neisseria meningitidis* utilization, adenosine guanosine, inosine
Pyelonephritis, experimental, mycoplasmosis, antibody
Pyelonephritis, *Mycoplasma arthritidis Escherichia coli*
Radioimmunoassay hepatitis B antigen, hepatitis B antibody *Staphylococcus aureus*
Radiotherapy lymphoma, cytotoxicity transformation
Resistance factors, *Escherichia coli* elimination, anthelmintics
Respiratory tract infection, viruses, diagnosis, fluorescent antibody technique
Rheumatoid factors, cross-reaction IgG
Rubellavirus, IgM-antibodies, IgA-antibodies, indirect immunofluorescence
Salmonella infantis intestines, chickens
Sarcoidosis, lymphocyte transformation, phytohaemagglutinin
Schizogony microgametogony *Toxoplasma gondii*, ultrastructure
Semliki Forest virus, mutation, viral RNA synthesis, arboviruses
Semliki Forest virus, virus replication, messenger ribonucleoprotein, viral nucleocapsid
Serotyping, immunoelectrophoresis, *Haemophilus influenzae*
Specificity antibody haptens, immune tolerance, immunologic memory
Spleen cells, mouse, amyloidosis, casein, cellular immunity
Stachybotrys alternans mycotoxin
Stachybotrys alternans mycotoxins, biological assays


9
PV
PL
N
G
N
IM


10
IM
G
PN
N


11
IM
PN
G
N


12
N
G
IM


13
PL
G
N
IM

Stachybotrys alternans mycotoxin, chromatography tissue culture toxicity 7
Stachybotrys alternans mycotoxins, pregnancy complications, mice 457
Staphylococcal protein A, macrophages 283
Staphylococcal protein A-reactive globulins, phylogeny birds, mammals 12
Staphylococcal protein A, *Staphylococcus aureus* 821
Staphylococci, cell wall, polysaccharides, animal 382
Staphylococcus aureus antigenic structure, phage pattern, horse strains 899
St phylococcus aureus hepatitis B antigen, hepatitis B antibody radioimmunoassay 422
Staphylococcus aureus immunization, antiglobulins 829
Staphylococcus aureus Pseudomonas aeruginosa, cystic fibrosis, epidemiology 541
Staphylococcus aureus staphylococcal protein A 821
Staphylococcus, cell wall protein A, fluorescent antibody technique, diagnosis, *Herpes simplex* 323
Stomatitis, recurrent aphthous, hypersensitivity humoral immunity immunofluorescence 107
Streptococcal groups A, B C, and G IgG uptake, quantitation 475
Streptococcal groups A, B, G, D and G agglutination, sheep red blood cells 19
Streptococcal groups A, B, C, D and G venereal diseases, urethra, cervix 470
Streptococcal groups A, C, G haemolysins and enzymes, isoelectric focusing 860
treptococcal groups B and D beta-glucuronidase 353
Streptococcus mitis aminopeptidase cell lysis 602
Streptococcus mitis hyaluronidase activity aminopeptidase activity 321
Streptococcus mitis hyaluronidase, aminopeptidase extraction 593
Streptococcus mitis hyaluronidase, aminopeptidase growing cells 608
St eptococcus mitis hyaluronidase, aminopeptidase, non-growing cells 613
Streptococcus mitis hyaluronidase repression 625
Streptococcus mutans endocarditis, dental plaque, properties 357
Streptomycin resistance penicillin resistance *Moraxella osloensis* 318
SV40-transformed cells, decreased oncogenic potential, diffusion chambers, peritoneal cavity 687
SV40-tumour tumour immunity diffusion chambers 676
Syphilis serodiagnosis, blood bactericidal activity treponema immobilization test 185
Thymidine kinase, thymidine phosphorylase, nucleoside deoxyribosyltransferase *Moraxella, Acinetobacter*
Thymidine kinase, thymidine phosphorylase, nucleoside deoxyribosyltransferase, *Neisseria*
Thymidine phosphorylase, nucleoside deoxyribosyltransferase thymidine kinase, *Moraxella Acinetobacter*
Thymidine phosphorylase, nucleoside deoxyribosyltransferase, thymidine kinase, *Neisseria*
Thymus, graft-versus-host reaction, amyloidosis, cortisol
Thymus, immune deficiency in mice, spontaneous cancer tumour immunology
Thymus-less mice, congenitally eosinophil response, migrating larvae, *Ascaris suum*
T-mycoplasmas, immunofluorescence, haemagglutination, polyacrylamide-gel electrophoresis
Tolerance, immune haptens, antibody specificity immunologic memory
Torulopsis glabrata, Candid albic as opportunistic infections
Toxoplasma gondii antibodies, immunoperoxidase test
Toxoplasma gondii, microgametogony schizogony ultrastructure
Transmission, transplacental, *Nosema cuniculi* blue fox, hypergammaglobulinaemia
Transplantation, bone marrow T cell precursors, cell fractionation, graft-versus-host reaction
Treponema calligyrum Treponem minutum Treponema microdentium ultrastructure
Treponema immobilization test, blood bactericidal activity syphilis serodiagnosis
Treponema microdenti m Treponem calli gyrum Treponema min tum ltra structure
Treponema minutum Treponema calligyrum Trepo ema microdentium ultrastructure
Trepo ema phagede is Treponem vincen tii Treponema refringens ultrastructure
Treponema refringens T eponem phagedenis, Treponem vincentii ultrastructure
Treponema vincentii Treponema phagedenis Treponema refringens ultrastructure
Tumour immunology immune deficiency ir mice, thymus, spontaneous cancer
Typing *Mycobacterium ranae (fortuitum)* bacteriophage
Ultrastructure *Toxoplasma gondii* microgametogony schizogony

that remains of the inner membrane of the pellicle of the microgametocyte

DISCUSSION

The wall forming the parasitophorous vacuole in the intestinal epithelial cells of the cat is much thicker and lacks microvilli compared with that forming the parasitophorous vacuole in other host cells. These microvilli were termed stereocilia by *Sheffield & Melton* (1968) This confirms the results of *Sheffield* (1970) for the intestinal forms of *Toxoplasma*

The trophozoite within the cat intestinal epithelium, undergoes dedifferentiation in a manner similar to *Eimeria tenella* and *E praecox* reported by *McLaren* (1969) and *Lee & Millard* (1971) respectively by losing the rhoptries and micronemes.

Prior to the initial nuclear division, we are unable to determine whether the trophozoite is committed to develop into a schizont microgametocyte or macrogametocyte. Once nuclear division has commenced the macrogametocyte can be excluded from consideration. In the majority of coccidians the number of microgametes exceeds that of the merozoites. Consequently microgametocytes, in the later stages of nuclear division, can be distinguished from schizonts on the basis of number of nuclei present. In addition at the later stages of development, the microgametocyte and schizont can be distinguished on the basis of their cytoplasmic organelles for example the schizont exhibits the conoid and developing rhoptries of the immature merozoites and the microgametocyte exhibits the flagella of the developing microgametes.

In the case of *Toxoplasma* it is unfortunate that the number of merozoites which develop approximate to the number of microgametes formed. Thus distinction between schizont and microgametocytes cannot be made on the basis of nuclear number Until the present time the distinction between schizonts and microgametocytes at the electron microscope level has been based on the presence of the cytoplasmic organelles However we now feel that we can distinguish between these organisms at an earlier stage of development on the basis of changes in nuclear structure, as described in the results (Fig 5–8)

In these multinucleate organisms, differences are already present which distinguish the schizont from the microgametocyte. We are of the opinion that schizonts can be characterised *by the nuclear structure which has the chromatin distributed in small patches* throughout the nucleus. They are similar to the appearance of the schizont nuclei of *Eimeria spp* as seen in the micrographs in papers by *Sheffield & Hammond* (1967) *Snigirevskaya* (1969b) *Sampson & Hammond* (1972) and *Roberts et al.* (1970) Similarly our opinion that the microgametocyte can be characterised by the nuclei having the chromatin distributed in large dense areas, predominantly at the periphery is in agreement with those seen in the micrographs of early microgametogony in *Eimeria spp.* as shown by *Cheissin* (1965) *Hammond et al.* (1967) and *McLaren* (1969)

Piekarski et al (1971) have proposed that daughter formation begins at the four nucleate stage We have not been able to confirm this finding since, in some of our sections, six nuclei are present at the commencement of daughter formation. On the basis

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Fig 21 Mature microgametocyte showing the microgametes lying free in the parasitophorous vacuole. × 16,000

Fig 22 Transverse section through anterior of a microgamete showing 2 flagella and a row of 4 microtubules. × 90,000

Fig 23 Transverse section of microgamete slightly posterior to that in Fig. 22 showing the nucleus, mitochondrion and row of 4 microtubules. Note what appears to be a unit membrane with an underlaying osmiophilic layer enclosing the organism. × 90,000

Fig 24 Longitudinal section of the anterior of a microgamete showing the perforatorium. × 32,000

Fig 25 Anterior of a microgamete showing the position of the basal body and flagellum enclosed by a unit membrane. Note the absence of the osmiophilic layer × 51,000

Ureaplasma urealyticum aminopeptidase 917
Vaccine protection, interferon, interferon production, viral infections 904
Venereal diseases, streptococcal groups A, B, C, D and G urethra, cervix 470
Venules, post-capillary lymphocyte traffic, *nude* mice, electron microscopy 249
Viral RNA synthesis, Semliki Forest virus, mutation, arboviruses 810
Virus B hepatitis, diagnosis 396
Virus replication, Semliki Forest virus, messenger ribonucleoprotein, viral nucleocapsid 53
Viruses, respiratory tract infection, diagnosis, fluorescent antibody technique 41
Worm burdens, faecal egg counts, *Ascaris suum* 879
Yersinia enterocolitica Yersinia pseudotuberculosis infection, antibodies 303
Yersinia pseudotuberculosis Yersinia enterocolitica infection, antibodies 303

SUPPLEMENTS

Supplement 244 — *Herbitz Thorsten B. & Haugen Olav A.* Endocrine Disturbances in Men with Benign Hyperplasia and Carcinoma of the Prostate. A Morphological Study in an Autopsy Series. Pp. 13 1974 (Section A)

Supplement 245 — *Hofer P A., Larsson P A Ek B Gåller H., Laurell, H & Lorentzon R* A Clinical and Histopathological Study of Twenty-Seven Cases of Urbach-Wiethe Disease. Dermatologic, Gastroenterologic, Neurophysiologic, Opthalmologic, and Roentgendiagnostic Aspects, as well as the Results of some Clinico-Chemical and Histochemical Examinations. Pp. 87 1974 (Section A)

Supplement 246 — *Krogh P Axelsen N H Elling, F., Gyrd-Hansen N Hald B., Hyldgaard Jensen J Larsen A E. Madsen, A Mortensen H P., Møller T Petersen, O K., Ravnskov U., Rostgaard M & Aalund O* Experimental Porcine Nephropathy Changes of Renal Function and Structure Induced by Ochratoxin A-Contaminated Feed. Pp. 21 1974 (Section A)

Supplement 247 — *Clemmesen Johannes* Statistical Studies in the Aetiology of Malignant Neoplasms. Pp. 266 1974 (Section A)

Supplement 248 — Dissertationes in honorem Ola Torgersen 1974 Pp. 180. 1974 (Section A)

Supplement 249 — *Olsen St en Petersen V Posborg, Hansen E. Sommer & Bohman S-O* Studies of Extracapillary Glomerulonephritis. Pp. 54 1974. (Section A)

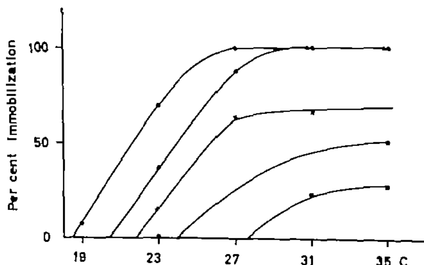


Fig. 5 Immobilization at various temperature, 19 –35 C and serum concentrations, 0.5 0.36, 0.25, 0.179 and 0.125 (Illustrated from left to right)

various dilutions of a stock suspension of treponemes were prepared.

The degree of treponemal immobilization caused by an undiluted human NS was recorded at various intervals during 120 minutes at three different treponemal concentrations (Fig. 4) After 120 minutes incubation, the titres of the NS were also determined at the various treponemal concentrations. The titrations were performed with and without extra complement being added to the serum dilutions.

As seen in Fig 4 the time for the reaction to reach the 50 per cent immobilization level varied inversely related with the treponemal concentration. At the highest concentration the immobilization curve did not reach the 100 per cent end point within the observation period The NS titres after 120 minutes incubation without extra complement added were found to be 16.0 11.2, and 4.0 residual complement being demonstrated in serum dilutions 1 16.0 1 16.0 and 1.5.6 at the treponemal concentrations 9.0 × 10^{3} 9.0 × 10^{6} and 9.0 × 10^{7} respectively

Extra complement was added to all the serum dilutions (0.125 ml serum (C160) dilution + 0.125 ml undiluted serum (C160) absorbed with treponemes at 0 C (to be published) + 0.25 treponemal suspension) to test whether in the present experiments in which relatively high treponemal concentrations were used complement might be a limiting factor At these controls, however the titres obtained were the same as those to be obtained when no extra complement was added.

According to the results obtained in the above experiments on the test procedure, a treponemal concentration as low as possible should be maintained. For practical purposes, 4.5 × 10^{6} treponemes per ml (five treponemes per microscopic field) were chosen. It was desirable to dilute the rabbit tissue material as much as possible in order to minimize any non-specific influence on the immobilizing activity Therefore, only testicles yielding at least 4.5 × 10^{7} treponemes per ml were accepted.

Immobilization at Various Temperatures

The immobilizing capacity of various dilutions of human NS was determined at different temperatures. As seen in Fig 5 the highest serum concentration tested caused 100 per cent immobilization at 27–35 C, whereas in the lower concentrations there seemed to be a maximal immobilization at 35 C At 39 C, as seen also in most experiments at

genase in enterobacteria (4) whereas in *Lactobacillus* this method was useful in classification (11)

6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase and 2 keto-3-deoxy-6-phosphogluconate aldolase are found in the same *Neisseria* species as are glucokinase and G-6-P dehydrogenase (7) The occurrence of these enzymes of carbohydrate metabolism in the "true neisserias" and their absence from the "false neisserias is another indication of the fundamental difference between these two groups of microbes.

The excellent technical assistance of Miss *Toril Johansen* is greatly appreciated. This investigation has been supported by a grant from *Norges almenvitenskapelige forskningsråd*

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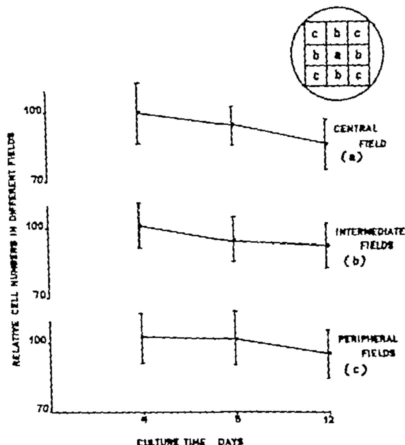


Fig. 6. Survival of human monocytes in different areas of the culture dishes. Each mark is the mean of the cell numbers in three different types of culture fields, expressed as the percentage of the cell number in the central field on the fourth day of culturing. The standard deviations are marked as bars. The cells in 18 culture dishes were counted

18.7 per cent. No significant relationship between the numbers of adhesive cells and the total number of leucocytes in the cell suspensions was found

Fig. 3 and 4 show the survival of the adherent cells, cultured on plastic surfaces, after 4 to 12 days culturing. A rapid decline in the cell numbers was observed in the first days of incubation. However from the fourth to the twelfth day of culturing the cell number fell only slightly

As shown in Fig. 5 no significant increase in the numbers of attached and surviving cells was found after 4 and 8 days culturing in medium containing greater volumes of serum. Cells cultured in medium without serum or with only 10 per cent serum were found to round up and fall off the plastic surface after a few hours of incubation. No sign of cell proliferation was observed in the cultures containing standard culture medium.

In order to evaluate the conditions for cell attachment and cell survival in different parts of the plastic dishes, the numbers of adherent cells were counted in different squares in the dishes as shown in Fig 6. There was no significant difference in the culturing conditions in different parts of the culture dish. The decline in cell numbers was equivalent to that shown in Fig. 4

In order to establish an even monolayer culture of adhesive phagocytes in the dishes at culture start it was considered important to maintain a uniform cell suspension dur

TABLE 1 *Results of Immunofluorescent Examination of Kidney Biopsies*

Number of patients and diagnosis*	Type of fluorescent conjugate						
	γ-glob.	IgG	IgA	IgM	IgE	C3	Fibrinogen
62 Chronic glomerulonephritis	58	58	46	37	–	60	30
4 Anti-GBM-nephritis**	4	4	2	2	–	4	?
6 Glomerulonephritis in transplants	6	5	2	1	–	6	5
4 Purpura nephritis	4	4	4	3	–	4	3
5 SLE nephritis	5	5	5	5	–	5	2
1 Membranous nephropathy	1	1	1	1	–	1	–
8 Idiopathic (lipoid) nephrosis	4	4	4	2	–	2	2
21 Haematuria and/or proteinuria only	11	10	9	10	–	14	6
6 Amyloidosis	6	6	6	6	2	6	4
2 Interstitial nephritis	–	–	–	–	–	–	1
119 Total	63	60	51	42	2	64	31

The diagnosis is based on clinical status, light microscopic examination and immunological findings.
** This group includes two cases of Goodpasture's syndrome.

RESULTS

The results are shown in Table 1. Immunoglobulins, mainly IgG and complement, were found in nearly all biopsies in which pathological lesions were visible in the light microscope. In the group of patients with haematuria and/or albuminuria, only about 50 per cent had deposits of IgG in their glomeruli. This was also the case of patients suffering from idiopathic nephrosis.

A striking feature of Table 1 is the almost complete negative results with the anti-IgE conjugate. Only two biopsies were definitely positive and they belong to the amyloidosis group. One dubious finding in a biopsy from a kidney with chronic glomerulonephritis could not be accepted as a clearly positive finding.

DISCUSSION

The results obtained with anti-IgG, anti-IgM, anti-C3 and anti-fibrinogen are in accordance with earlier reports (10–13) but, in contrast, IgA was found in a relatively high proportion of the cases. These results will be presented in detail and discussed in a forthcoming paper. It should be noted that as many as about 50 per cent of the cases of non-symptomatic haematuria and/or albuminuria had deposits of various proteins. The main object of this study, however, was to investigate whether deposits of IgE could be demonstrated in patients with kidney disease, especially in those with various types of nephrotic syndrome, as described by *Gerber & Paronetto* (8). In their series comprising 13 patients with different types of nephrotic syndrome, IgE deposits were found in 9 cases. Only cases of nephrotic syndrome with minimal changes (idiopathic nephrosis) treated with steroids were negative or weakly positive. Moreover, the amount of IgE could be correlated to the degree of proteinuria. In our series, however, which like that of *Gerber & Paronetto* comprised cases of treated and non treated idiopathic nephrosis, membranous nephropathy and chronic glomerulonephritis, no IgE could be found except in patients with amyloidosis. We have no reason to doubt the quality of the anti-IgE conjugate used in our experiments since it was shown to be monospecific by the test with standard particles and also suitable for investigating skin biopsies and serum from patients with bullous pemphigoid. It is therefore rather difficult to explain the discrepancy between our respective findings. On the other



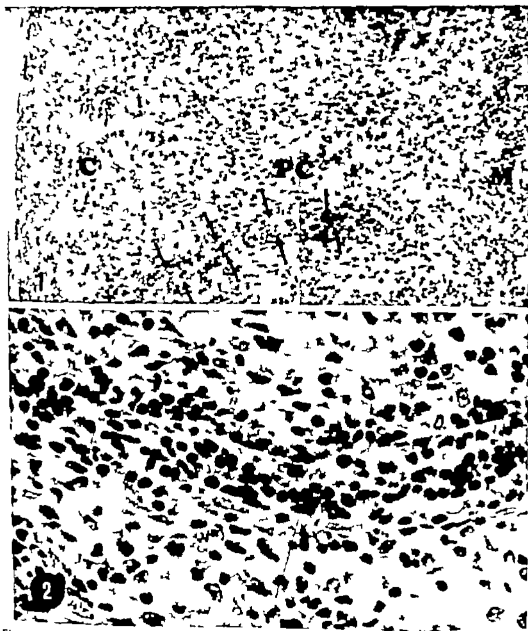


Fig. 1 One micron section of an inguinal lymph node fixed in 2.5 per cent glutaraldehyde from a nu/nu mouse. Note severe depletion of free lymphoid cells in both the paracortical and cortical areas. A large post-capillary venule is seen (arrows). C: cortex, PC: paracortex, M: medulla.

1 per cent toluidine blue × 180

Fig. 2 One micron section of large post-capillary venule from the lymphocyte depleted paracortical area of nu/nu mouse lymph node. Note the numerous leucocytes traversing the vessel wall.

1 per cent toluidine blue × 700

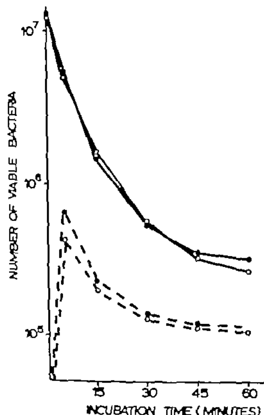


Fig 3 Number of viable total (———) and intracellular (– – – – –) *bacteria during incubation of* leucocyte-bacteria suspensions containing leucocytes preincubated with (●) and without (○) phenylbutazone (mean of five experiments)

eliminated by antibiotics which cause no inactivation of phagocytized bacteria (8) Killing of extracellular bacteria takes *however* 10–15 minutes. During this period inactivation of intracellular bacteria by granulocyte enzymes may significantly obscure the results. Accordingly abrupt inhibition of the bactericidal activity of the granulocytes is a prerequisite for the measurement of the number of viable intracellular bacteria while extracellular killing by antibiotics takes place. As demonstrated in the present study this inhibition may be achieved by phenylbutazone. The essence of our method is, therefore the combined use of phenylbutazone for the inhibition of intracellular killing of bacteria and antibiotics for the control of *extracellular* bacteria.

The inhibition by phenylbutazone of the bactericidal activity of the granulocytes seems to be influenced not only by the drug concentration as demonstrated in the present study *but also* by the concentration of bacteria in the leucocyte—bacteria suspension. In the present study a slight reduction in viable bacteria was observed in the tests containing 2 4 and even 6 mg phenylbutazone especially during the later phase of incubation, indicating that complete inhibition of intracellular killing was not achieved. Most likely this was due to the high concentration of bacteria (1.5×10^7 bacteria per ml) in the test suspension. In previous studies, when only 10 per cent of the *present bacterial concentration* was used or even less, as in the suspension used for the determination of the number of viable intracellular bacteria, complete inhibition of bactericidal activity was achieved by 2 mg phenylbutazone per ml leucocyte-bacteria suspension (7 8)

The reason for the reduced phagocytic activity of the granulocytes in the presence of phenylbutazone remains obscure. For significant phagocytosis to occur attachment of opsonized bacteria to receptors on the phagocytic cell membrane by the Fc part of specific antibody molecules is required (3 5 12) Once attachment occurs, microprojections from the phagocyte membrane surround the bacterium and a phagocytic vacuole is formed. The energy required for this ingestion process *seems mainly to* be derived from anaerobic glycolysis (4 10) Interference by phenylbutazone with the Fab-or Fc *fragments of the* antibody molecules or with the receptors on the phagocytic cell surface might all result in reduced phagocytosis as well as impaired glycolysis. However in the presence of phenylbutazone, reduced glycolytic activity has been demonstrated in homogenates of guinea pig polymorphonuclear leucocytes (10) indicating that direct interference with the glycolytic system of the cells *might* be a more plausible explanation for the reduced phagocytic activity caused by phenylbutazone than interference with extracellular factors or receptors on the cell surface.

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STUDIES ON *STACHYBOTRYS ALTERNANS*

II Occurrence Morphology and Toxigenicity

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Isolation of 73 *Stachybotrys alternans* strains from various materials, their morphological description and results from toxigenicity tests by a tissue culture method are reported. The isolation method consisted of culturing the material on wet cotton wool and sterile filter paper. Out of the 725 samples of cereal grain, field pea, oat meal, feed and fodder sent for routine mycological investigation 50 strains of *S. alternans* were isolated. Rather strikingly approximately every fourth of the samples of field pea seed was found to be contaminated with *S. alternans*. Among the 129 samples of feed and fodder suspected as a cause of mycotoxicosis in domestic animals, 22 turned out to be contaminated by *S. alternans*. The toxicity of ether soluble extracts from strains cultivated on wheat-oats-barley mixture was tested in mouse primary fibroblast cultures. Together 49 out of the 73 isolated strains were found toxigenic by the test. All 13 strains isolated from field pea samples produced toxin. No relation between morphological characteristics and toxicity was revealed.

Stachybotrys fungi have often been reported in East European literature as a cause of mycotoxicoses in animals and occasionally in humans, whereas in Western mycotoxin literature these fungi have passed nearly unnoticed. The frequent occurrence of *Stachybotrys* fungi in Finnish grains and feeds (*Korpinen* 1973) has brought up the question about their possible toxic role as regards not only animals but also humans. The classical way for studying the toxicity of *Stachybotrys* fungi is to feed suspect or artificially infected material to experimental animals. Other methods common in mycotoxin studies are to apply an extract of suspect material to rabbit skin, to inject such material into experimental animals or to mix it with *Paramecium* suspension (*Spesivtseva* 1964). Tissue culture techniques have gained a steadily increasing importance in toxicology (*Rofe* 1971) including mycotoxin studies (*Umeda et al.* 1970).

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The morphologic characteristics (*Bisby* 1943) and the toxigenicity (*Bilaj & Pidoplitshko* 1970) of *Stachybotrys* fungi have been studied by several authors. The mycological taxonomy however is still obscure with several synonyms, e.g., *Stachybotrys atra* Corda, *S. alternans* Bon. *S. chartarum* (*Ehrenberg* ex *Link*) Hughes. This report describes the sources and the morphology of the 73 strains of *Stachybotrys* found in Finnish material and gives the results concerning their toxigenicity as studied in tissue culture.

MATERIAL AND METHODS

A. Isolation and Subsequent Cultivation of the Fungus

Isolation was attempted from various materials, which included seed samples of cereal grains, field pea, grass, clover, oat meal, mixed feed, hay, straw and silage. Studies were carried out during the years 1970-72 in addition to which one isolation was made in 1968. Part of the material consisted of 129 samples of feed and fodder suspected to be responsible

pling between the MI of the two groups did not occur

At PPD concentrations of 25 and 100 μg/ml, the distinction was less pronounced, high antigen concentration resulting in migration inhibition of nonsensitized cells. Puromycin did not influence the low MI of these cells in contrast to the effect observed if puromycin was added to cells from tuberculin positive persons at PPD 100 μg/ml.

Judging from these findings, the migration inhibition found at PPD 100 μg/ml of cells from tuberculin negative persons is independent of protein synthesis suggesting a different mechanism of migration inhibition.

Mann et al. (16) who used the capillary technique suggested that antigen toxicity could be disclosed by the inability of puromycin to prevent migration inhibition of non sensitized cells. Our findings seem to point to the same conclusion. This is further supported by the observation that the inhibition of these cells persisted after 48–72 hours, while the inhibition of cells from tuberculin positive persons was time limited and had disappeared after 48 hours. This may indicate that the toxic concentration of antigen is different for cells from sensitized and non-sensitized persons. If so, problems arise as to the appropriate level of antigen, since it is desirable to work with antigen concentrations just sub-toxic to sensitized cells to maximate the sensitivity of the test. False positive migration inhibition may reveal itself in the indirect migration inhibition assay (5) which has the disadvantage of being time consuming and technically difficult in work with soluble antigen. Instead, puromycin treatment and observation of migration areas during prolonged incubation may solve the problem involved in the fact that false positive results may be recorded as true immunological inhibition.

The present investigation demonstrates that this procedure functions with PPD induced migration inhibition and it may also be applicable to other antigens.

The study was aided by grants from the *Danish Medical Research Council.*

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for 65 alleged outbreaks or cases of mycotoxicosis in domestic animals. The remaining part was composed of 725 samples received by the Department of Plant Pathology of the Agricultural Research Centre for a routine mycological examination (Table 1). It should be pointed out that the latter material was not selected because of its moldy appearance but was collected by random sampling of normal field or storage material.

For isolation, the material was cultivated on wet cotton wool and sterile filter paper in large plastic dishes for 3 weeks. The dishes were kept in darkness at room temperature (18–20° C). The fungal growth was first observed by the aid of a stereomicroscope.

Pure cultures of *Stachybotrys* strains were started from the hyphal tips and grown on oatmeal agar. The substrate had the following composition:

oatmeal	50 g
agar	10 g
glycerol	5 ml
lactic acid	15 drops
distilled water to make	1000 ml

In order to test the toxigenicity of the fungus strains, a grain mixture composed of 1/3 wheat, 1/3 oats, and 1/3 barley was inoculated with the fungus mycelium grown in pure cultures on the above substrate for 2 weeks. This was done in Roux flasks, and the cultures were kept at room temperature (18–22 C) for 2 weeks, transferred into 0 C for 2 days, and once more kept at room temperature for 2 weeks. At the end of this incubation period the grains were covered by a black layer of the fungus.

B. *Morphological Studies*

For the observations of morphological details and the measurements of conidia and conidiophores of the *Stachybotrys* strains a compound microscope was used, slides made in lactic acid from 2 weeks old pure cultures serving as objects of study.

C. *Preparation of Inocula for Toxicity Test*

Each fungus-infected grain sample was dried at 55 C for 2–3 days. The grain sample was then ground in a Waring Blendor and 25 g of it was extracted with diethylether in a Soxhlet apparatus for 6–8 hours. The ether extract was concentrated about 25 ml and a toxic substance was precipitated from it with petroleum ether (b.p. 60–80 C) and further treated according to *Fialkov* and *Serebrianij's* method as modified by *Palyusik* (1970).

A few milligrams (5–30 mg) of the prepared powder were dissolved in 0.2 ml of 96 per cent ethanol and 2 ml water was added. The solution was brownish dark brown, milky or slightly opalescent. The alcohol-water solution was sterilized through a Millipore filter with 0.2 μ pore size.

D. *Tissue Culture Toxicity Test*

The toxicity of the substance prepared from each *Stachybotrys* strain was tested in primary fibroblast culture of the mouse foetus.

Foetuses were removed from ether anaesthetized mice in 16–19 days pregnancy. Preparation of cell suspensions and cultivation of monolayers took place according to standard methods (*Paul* 1972) with the following particular details. Trypsin at 1 per cent was used. The growth medium was Eagles minimum essential medium, glutamine 0.03 per cent, foetal calf serum 10 per cent, penicillin 100 I.U./ml, and streptomycin 100 μg/ml. Of the cell suspension in the growth medium 2.5 ml were placed in petri dishes 2.5 cm in diameter having a 13 × 13 mm cover slip on the bottom. From the foetuses of one mouse 20–30 tissue culture dishes could be prepared. The petri dishes were kept 24 hours at 37 C temperature in an atmosphere with 5 per cent CO_2.

The cell monolayers were washed with normal saline prior to inoculation. 0.1 ml of the solution to be tested, in 2.5 ml of the growth medium, served as inoculum. The cultures were further incubated at 37 C in 5 per cent CO_2 for another 24 hours and the effect on the cells was examined microscopically either without staining or after staining with May-Grünwald and Giemsa.

RESULTS

A. *Occurrence of* Stachybotrys *Strains*

Altogether 73 strains of *Stachybotrys* fungi were isolated. Of these 23 originated from materials suspected to be a source of mycotoxicosis, the remaining 50 were found in the material sent in for routine investigation.

Table 1 shows that all but one of the strains isolated from the routine material originated either from samples of cereal grains (44 per cent), from field pea (26 per cent) or from grass (22 per cent). The frequency of isolates among materials of which a relatively large number of samples was tested was highest in the field pea seeds (19 per cent) and somewhat lower in grass seed: meadow fescue, orchard grass and rye grass, (10 per cent) and in barley seeds (5 per cent). Of all routine samples examined for *Stachybotrys* during the years of 1970–72, 7 per cent

Transformation Methods

The principles of mutant selection, preparation of transforming DNA and transformation techniques have been described previously by *Bøvre* (3 4) and *Bøvre & Holten* (8)

The strains H102 and H161 grew well in the presence of 500 µg streptomycin per ml of blood agar medium. Accordingly transforming DNA was prepared directly from the wild types of these strains.

RESULTS

Morphological Cultural and Biochemical Investigations

According to the microscopic picture, the two strains consisted of short and plump Gram-negative rods with obtuse rounded ends somewhat resistant to decolorization. A tendency to occur in pairs was noted but somewhat less marked than in usual strains. Neither capsules nor endospores were observed. Motility was negative at 37 C and 22 C.

The strains grew slowly after overnight incubation at 37 C on 5 per cent horse blood agar colonies were rather small though their diameters enlarged up to 0 8–1.2 mm after 48 h. The colonies were low convex with a circular periphery more or less translucent to slightly opaque, unpigmented and glistening, and of a soft consistency No haemolysis was observed around isolated colonies, but a slight partial, greenish haemodigestion was noticed around the mass growth. Growth was not improved by incubation in a CO_2-atmosphere and a comparatively good growth was obtained on simple nutrient agar No growth occurred on Mac Conkey's agar nor in anaerobic conditions. In stab culture in semisolid media the strains grew on the surface and 2–3 mm below it after 48 hours at 37 C. No growth was observed in Heart Infusion Broth containing 6.5 per cent and 2.5 per cent NaCl. After growth for two days in Heart Infusion Broth the medium was moderately turbid and a flaky deposit was noticed as regards strain H102, a thin granulous pellicle on the surface, with a tendency to rise up along the wall of the tube, was noted. Growth in peptone media, including the MR VP medium, was poor Growth occurred in Kligler Iron Agar slants, with no change at the butt.

The oxidase reaction was positive both with dimethyl- and tetramethyl-p-phenylenediamine. The catalase reaction was positive. Nitrates were reduced to nitrites in fresh isolates (somewhat more strongly marked in strain H102) but this property was lost in subcultures Nitrogen gas production was not detected with inverted Durham tubes.

The following tests gave negative results. urease, indole, liquefaction of gelatin and coagulated horse serum, growth on Simmons citrate medium, methyl red and production of acetylmethylcarbinol, deamination of phenylalanine and tryptophan tyrosinase activity and opalescence on egg yolk agar acid production from glucose, fructose, galactose, arabinose and maltose

The strains were able to grow on the surface of Hugh & Leifson's O/F medium, without acid production. The strains utilized acetate as sole source of carbon on the basal medium as well as DL-β hydroxybutyrate. Sudan Black B staining of the bacteria cultured on the last mentioned medium revealed the presence of poly-β hydroxybutyrate inclusion granules in each of the two strains.

Antibiotic Sensitivity Testing

The strains were resistant to penicillin, streptomycin, sulphonamide oxacillin and lincomycin. They were sensitive to erythromycin, tetracycline, oleandomycin chloramphenicol, cephalotin, neomycin, gentamycin, colimycin, carbenicillin, kanamycin and the combination trimetoprim-sulphametoxazole. As regards ampicillin and novobiocin, intermediary sensitivity was noted.

The minimum inhibitory concentration of penicillin G ampicillin and tetracycline gave the following results for the two strains

Penicillin G 6.25 µg/ml (approximately 10 I.u./ml)
Ampicillin 3 12 µg/ml
Tetracycline 0.39 µg/ml.

TABLE 1 *Occurrence of Toxic and Non-toxic Stachybotrys Strains in Various Samples Received for Mycological Investigations in 1970–1972*

Material	Routine mycological investigation			Suspected mycotoxicoses		
	No. of samples investigated	No. of *Stachybotrys* isolates	No. of toxic/non-toxic isolates	No. of samples investigated	No. of *Stachybotrys* isolates	No. of toxic/non-toxic isolates
Barley	265	12	8/4	23	—	—
Oats	39	1	0/1	18	2	1/1
Wheat	13	6	1/5		—	—
Rye	7	3	2/1	—	—	—
Field pea seed	68	13	13/0	—	—	—
Grass seed	106	11	10/1	—	—	—
Clover seed	14	3	3/0	—	—	—
Oat meal	17	0	—	9	4	1/3
Mixed feed	140	0	—	44	4	3/1
Hay	25	0	—	17	7	5/2
Straw	1	1	0/1	4	3	0/3
Silage	30	0	—	12	2	1/1
Total	725	50	37/13	129	22	11/11

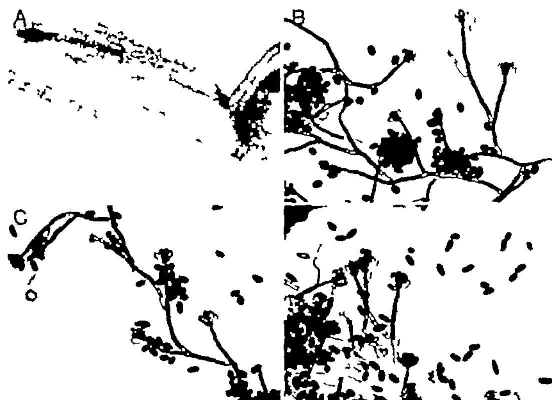


Fig 1 (A) Conidial heads of *Stachybotrys* on grass seed × 8.
(B): Toxic strain from cereal seed, size of conidia 8.65 × 5.77 μ × 250.
(C) Non-toxic strain from cereal seed size of conidia 9.77 × 6.79 μ × 250
(D) Toxic strain from field pea seed size of conidia 10.40 × 5.21 μ × 250.

Genetic Relations

Genetic transformation experiments were performed using streptomycin resistance as marker. Initially a semi-quantitative method introduced by *Bøvre & Holten* (8) was used. A suspension of the streptomycin sensitive wild type of strain 5873 (about 10^{8} cells per ml) was exposed to transforming DNAs from a streptomycin resistant mutant of strain A 1920 and from the wild types (primarily streptomycin resistant) of strains H102 and H161. Streptomycin was added from below after 8½ hours growth at 33° C. After three days there was heavy growth (more than 10^{4} transformants per plate) on all three parallels and no growth on the parallel without DNA. To obtain more accurate information, a quantitative method was used (8). The results of one experiment are tabulated in Table 1. The transformant yield was of the same order of magnitude whether DNA from the known *M. osloensis* strain or from the two new strains were used.

TABLE I. *Interstrain Transformation between Known and Suspected Strains of Moraxella osloensis*

Recipient strain, *Moraxella osloensis* 5873	
Donor strain	Transformants per ml
M. osloensis A 1920	9.8 × 10[illegible]
H 102	20 × 10[illegible]
H 161	8.5 × 10[illegible]

Quantitative streptomycin transformation was used as previously described by *Bøvre* (3, 4) and *Bøvre & Holten* (8). Identical samples of the streptomycin sensitive recipients (1.2×10^{8} *cells/ml*) were exposed simultaneously to the respective donor DNA samples (20 μg/ml). Following DNA exposure for 20 min at 33° C, DNase was added.

One tenth ml aliquots of appropriate dilutions were spread in duplicate on blood agar plates. The plates were incubated for about 6 hours. Then, streptomycin was added from below the agar followed by continued incubation for about 3 days before assay of transformants.

The donor mutant of strain A 1920 had been selected at 300 μg streptomycin/ml; the transformants were assayed at 50 μg/ml. The strains H102 and H161 grew primarily in the presence of 500 μg streptomycin per ml. Accordingly, no mutant selection was necessary before preparation of transforming DNA from these two strains.

One parallel without DNA gave no growth, i.e. no spontaneous mutations were observed.

DISCUSSION

As regards most of the conventional criteria, the two strains H102 and H161 appeared to be identical with *M. osloensis* but they differed from previously described strains in being resistant to penicillin and to streptomycin, whereas previously known strains have been fairly sensitive to penicillin (2, 6, 11, 12, 13, 19) and highly sensitive to streptomycin (6).

Baumann et al. (2) even included penicillin sensitivity as a genetic characteristic of the genus *Moraxella* ("none can grow in the presence of 1 unit of penicillin G per milliliter"). These differences indicated that it would be desirable to verify the identity of these strains by genetic transformation experiments using authentic strains of *M. osloensis*. The results of such experiments prove beyond doubt that the two strains are indeed *M. osloensis*.

Although *Snell et al.* discovered in the United Kingdom growth of three strains of *M. phenylpyrouvica* in the presence of 100 I.U. penicillin/ml (20), penicillin resistant strains of *M. osloensis* have, as far as we are informed, never been mentioned and the present strains may thus be considered the first to be reported.

As previously pointed out by *Snell et al.* (20), penicillin sensitivity as a criterion for inclusion in the genus *Moraxella* should not be considered an absolute requirement.

The high resistance to penicillin and streptomycin of the two strains raised the question whether they could be mutants selected by antibiotic treatment. In the first case, none of these antibiotics was given before specimens for bacteriological examination had been taken. Thus, the treatment of this patient does not explain the resistance. In the second case, treatment with penicillin was

TABLE 2. *Toxicity of Stachybotrys Strains Isolated in Connection with Suspected Outbreaks of Mycotoxicoses*

Material from which isolated	Number of outbreaks	Main symptoms	Animal species affected	Number of toxic/non-toxic isolates
Hay	7	Respiratory symptoms (2 herds)	Cattle	1/1
		Fever enteritis (2 herds)	Cattle	2/0
		Paresis, liver degeneration (2 herds)	Cattle	2/0
		Enteritis (zoo animals)	European elk	1/0
Straw (bedding)	3	Unspecified high mortality (2 farms)	Poultry	0/2
		Respiratory symptoms (1 herd)	Cattle	0/1
Silage	2	Paresis, liver degeneration (2 herds)	Cattle	1/1
Oats	6	Sterility (3 herds)	Cattle	0/3
		Retarded growth (2 farms)	Swine	2/0
		Respiratory symptoms (1 herd)	Cattle	0/1
Mixed feed	5	Sterility (2 farms)	Swine	1/1
		Gastrointestinal symptoms (1 farm)	Swine	0/1
		Unspecified high mortality (1 farm)	Poultry	1/0
		Fever agalactia (1 farm)	Swine	1/0
Total	23			12/11

= isolated in 1968.

were found to be contaminated by this fungus.

B. *Morphological Data of the Fungus*

The strains isolated could be morphologically widely different as regards several details. The following description gives the range of variation of the properties observed and measured.

Stachybotrys growing on seeds was easily recognized by its black, rounded heads of conidia (Fig 1A) On oatmeal culture it formed colonies growing close to the agar surface. The mycelium was white at first, darkening with increasing age, and at the end of 2 weeks it was almost black with production of spores. The spores developed very rapidly

The hyphae were hyaline 2–4 μ in thickness. The conidiophores were 30–70 μ long and 3–5 μ wide with a few septa simple or sympodially branched The upper portion, usually darker beared a group of phialides (sterigmata) The phialides were hyaline or dark coloured, 7–12 μ long and 3–5 wide their number varying from 6 to 10 The conidia, which were produced at the small tips of the phialides, were greatly variable in size 6–14 μ × 4–9 μ, averaging 9.3 μ × 6.2 μ. The conidia were oval or rounded hyaline or dark-coloured smooth walled or occasionally showing short spiny structures. Young conidia were often guttulate (Fig 1B-D)

C *Toxicity to Cell Culture*

Out of the 73 *Stachybotrys* strains isolated, 49 were found to be toxigenic under the conditions of the present study The frequency of toxic strains in the material sent in for routine mycological investigation is stated in Table 1 The corresponding data for material suspected to be responsible for mycotoxicosis are presented in Tables 1 and 2

The extracts from 49 strains regarded as toxic in the conditions of the present test all caused total destruction of the respective cell culture during the 24-hour incubation period The strains considered non-toxic had no ob-

have been isolated from hamster rat and man and have not been encountered in connection with subacute endocarditis. *S. mutans var. Clarke* comprises such biotype 1 strains as belong to serotype c and genotype I of Coykendall. They have all been isolated from man and they are the strains that together with the biotype 1 strains belonging to serotypes e and f are isolated from subacute endocarditis as well as from dental plaques and processes of caries. *S. mutans var. rattus* belong to biotype 2, serotype b and genotype II. They have been isolated from plaques and caries of the rat as well as from dental plaques of man. They are highly cariogenic in the rat (17, 32). Finally biotype 3 strains belong to serotype d, g or SL–1, the latter represents *Coykendall's S. mutans var. SI* and belong to his genotype III. They have not been encountered in connection with subacute endocarditis but they are frequently isolated from carious processes and dental plaques of man.

We wish to thank *J. Henriksen*, who has reviewed the manuscript, *F. Lichtenberg, C. one, K. Lyngaard*, *H. dam* and *M. Kilian* for providing us with the samples from carious lesions and dental plaques. Thanks are due also to *D. Bratthall, G. Colman, A. L. Coykendall, R. J. Fitzgerald, B. Guggenheim* and *J. M. Jablon* and *D. D. Zinner* for the kindness with which our inquiries for strains have been met.

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servable effect during the same period as judged by comparison with control cultures.

D Relation of Morphological and Toxic Properties

As reported above, extensive variations were observed among the isolates in certain morphological properties, especially in the colour of the mycelium grown on the grain substrate and in the conidium size. No correlation of any morphological feature to toxicity or non toxicity was demonstrated.

DISCUSSION

Stachybotrys fungi are slow growers (*Bisby* 1943 *Danko* & *Tanyi* 1968) This impedes efforts of isolation from field material. On nutrient material the fungus often escapes notice owing to overgrowth of other more competitive fungus species. The method applied in this work was based on the use of the filter paper which is known for its selective properties with regard to *Stachybotrys* and *Dendrodochium* fungi (*Spesivtseva* 1964) Whether this method could reveal all those samples contaminated with *Stachybotrys* is not known, however By using ultraviolet light the abundant overgrowth of many fungus species may also be prevented thus providing room for *Stachybotrys* growth. The latter method was used in an earlier work (*Korpinen* 1973) In the present work the filter paper method was chosen mainly for practical reasons. The optimum circumstances for isolation of *Stachybotrys* fungi from different materials should be studied.

By the isolation method used 50 (7 per cent) out of 725 samples tested proved positive in the routine mycological investigation. The material is larger than in any earlier report outside Eastern Europe The frequency is unexpectedly high, especially as the occurrence of *Stachybotrys* fungus outside Eastern Europe has been barely known. Of particular interest is the fact that all but one of the strains originated from the seeds of various cultivated plants Several authors have emphasized the fact that *Stachybotrys* fungi grow mainly on materials rich in cellulose, such as hay or straw (*Forgacs et al.* 1958, *Mirocha et al.* 1972 *Spesivtseva* 1964) *Malone* & *Muskett* (1964) and *Mäkelä* (1972) have reported *Stachybotrys* also on oats seeds and grass seeds, respectively The frequent occurrence of the fungus on seeds suggests a threat for human as well as animal health unrecognized heretofore.

In the material suspected as sources of mycotoxicosis in field conditions, the frequency of *Stachybotrys* was a little higher (17 per cent) than in the material just discussed. This fact suggests that the said material could be selected which supports the diagnosis of stachybotrytoxicosis in at least part of the suspected cases of outbreaks. Because the clinical and epidemiological suspicion of stachybotrytoxicosis is as vague as it is and in view of possible involvement of other mycotoxicoses, the frequency of isolates in the material concerned is difficult to assess.

According to the literature concerning tests for toxicity of *Stachybotrys* the rabbit skin test has been most often applied. *Bodon* & *Palyusik* (1970) claimed that the tissue culture toxicity test for *Stachybotrys* is superior in sensitivity to other biological tests. This and other advantages induced us to develop a tissue culture test for the purposes of the present and future studies.

The proportion of the toxic strains among all the isolates was 66 per cent. This largely agrees with the percentages found by *Forgacs et al* (1958) The result also offers proof of the sensitivity of the tissue culture test used. Of particular interest is the fact that all 13 isolates from the field pea, which is also used for human consumption, were found to be toxin producers.

In an earlier study (*Korpinen* 1973) 9 out of 19 strains tested were found by a feeding test with mice to be toxic The material was included in the present tissue culture toxicity tests. Two out of its 9 strains turned out to be negative under the conditions of the test no cell destruction was observed within 24 hours. When these strains were subsequently subjected to more prolonged observation,

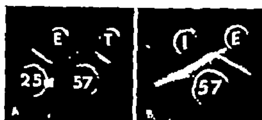


Fig 1 Characterisation of antisera nos. 57 and 25 by immunodiffusion in agarose gel.

Well 57: Antiserum no. 57 with activity against CEA and NCA, absorbed with *normal human serum* and PCA extract of normal liver

Well 25a Antiserum no. 25 absorbed with normal human serum, possesses activity against β_E and an associated α-protein.

Well T: Perchloric acid (PCA) extract of colonic carcinoma.

Well E Sephadex G-200 *fraction* (2. peak) of PCA extract of colonic carcinoma, contains β_E (NCA) and α-protein.

Well I *Sephadex G-200 fraction (1 peak)* of PCA extract of colonic carcinoma *contains β_I*-protein (CEA)

A) Note the β_I-line (CEA) (closest to the well T) the β_E-line (NCA) (closest to well 57) and further the *β_E-line (NCA)* against fraction E and the α-line between antiserum no 25a and excess of α-protein in the absorbed antiserum no. 57 The single line between antiserum no. 25a and fraction E suggest *cross-reaction* between the β_E and α-protein.

B) Cross-reaction between the β_I and β_E-protein is seen. The cross-reacting antigen is referred to as non-specific cross-reacting antigen (NCA) In this test system, NCA is defined by the β_E line and CEA is defined by the β_I-line.

ther absorption was done with human red cells of group A and B until no agglutination occurred with these cells. I gel-diffusion studies against extract of colonic carcinoma, *two distinct* precipitin lines were seen (Fig. 1A, well T) The two lines are referred to as the β_I-line, closest to the T well, and the β_E-line The *β_I-line* showed a reaction of complete identity with the CEA-line *given by* an anti-CEA serum provided by *GOLD* (21) Reaction of partial identity between the corresponding β_I and β_E-proteins was found, as demonstrated in Fig. 1B. The antigens shared by these two proteins are referred to as non-specific cross-reacting antigens or NCA.

Antisera nos. 57 and 14 did not react with the α-protein which seemed to share antigenic determinants with the β_E-protein. In fact, an excess of this α-protein was demonstrated in the absorbed antisera, as seen in Fig. 1A, where antiserum 25 gives a precipitin line with protein added to serum 57 for absorption purpose. This protein was found to have an electrophoretic mobility close to that of α-antitrypsin, as indicated in Fig 5

Antisera nos. 25 and 26 showed identical reactions and were used for further study of the α-protein. These antisera were produced in rabbits immunized with PCA extract of colonic carcinoma eluted in the second peak from a Sephadex G-200 column. The unabsorbed *antisera had strong* activity against albumin and α-antitrypsin (Fig. 5) which was removed by absorption with 0.2 ml *normal human serum* per ml antiserum. The absorbed antisera show reaction with *two proteins* (Fig 4 well T) one of which is the α-protein, the other was identified as the β_E-protein. With *antiserum 25 and 26* a reaction of partial identity between the α- and β_E-protein appeared, as seen in Fig. 5 A reaction of partial identity is also indicated in Fig. 1A between the α-protein in well 57 and the *precipitated* β_E-protein between well E and 57

RESULTS

Identical results were obtained with antisera nos. 14 and 57 CEA as well as NCA was demonstrated in normal colonic mucosa (nc) and in mucosa from ulcerative colitis (uc) (*Fig* 2A) in normal liver (Fig 2B L3) *normal* urine (U7) and urine from a cancer patient (*U6*) (*Fig.* 2C) saliva (Fig 2D,s2) and in blood *group substance* A (Fig 2E) CEA could not be demonstrated *by* our methods in normal human serum (Fig 2A and Fig 2C, S1 and S4) nor in the H and Le^a substances tested (Fig 2E) CEA was not found in the porcine and equine AB specific *substance*.

One *of the* pulmonary tumours (Fig 2F PT) was CEA positive the other was negative, both extracts were tested at protein concentrations of 40–45 mg/ml.

The results are summarized in Table 1 Positive CEA reaction was found in extracts of normal liver and colonic mucosa from four patients *died* of diseases other than cancer No differences were observed when the CEA reactions were *compared* with the reactions of normal tissue from *four* patients who died of cancer in remote organs. *Positive CEA* reactions were found in normal tissue *extracts*

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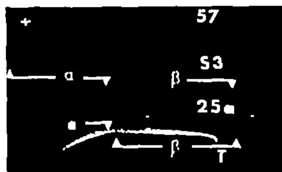


Fig 4 Detection of the α-protein in tumour extract and normal serum by immunoelectrophoresis in agarose gel.

Trough 25a: Antiserum no. 25 absorbed with normal serum to remove activity against albumin and α_1 antitrypsin present in the unabsorbed antiserum (Fig. 5)

Trough 57 Primarily absorbed antiserum no. 57

Well S3 Perchloric acid extract of normal serum, S3

Well T Perchloric acid extract of colonic carcinoma.

Reaction of complete identity between the β_E and α-line is seen when PCA extract of normal serum S3 is tested against antiserum 25a. The α-protein present in excess in antiserum 57 gives a precipitin line with antibodies in serum 25a. This line which runs parallel with the antisera troughs shows reaction of complete identity with the double arched α-β_E-line. Against tumour extract, T spur formation is seen. The anti NCA activity in serum no. 57 was too weak to precipitate the β_E-protein in this experiment. (The anodic part of the β-arch is not visible on the reproduction)

26 The α protein was found in all the samples of colonic mucosa and liver tissue and in all the normal sera. It was found in the cancer tissue extracts and in urines from cancer patients, (Fig 4) By our method it was not detected in the two normal urines, nor in the samples of saliva. This may however be due to the rather low sensitivity of the method used for these studies. Study of the α protein was done after preliminary observations which *suggested that some association* between the α and β_E-protein could exist. Our data were therefore limited to the demonstration of this protein and to show its reaction pattern in some gel-diffusion experiments. Distinct spurformation between the α and β_E-protein was seen in tests with some tissue extracts (Fig 4 well T) while other extracts of tissue serum and urine showed a long double arched precipitin line with no spurring (Fig 4 well S3) The two arches correspond to those of the α and β_E-precipitin lines.

DISCUSSION

Our study shows that CEA as well as NCA are present in small amounts in apparently normal colonic mucosa and liver Similar findings have recently been reported by others who by immunodiffusion methods have demonstrated CEA or CEA-like material in normal colon extract (18) and in normal lung and breast tissue (15) No tumour specific CEA variants could be distinguished by our antisera. The existence of tumour specific determinants can not be excluded, however. In fact, the reaction of partial identity with tumour CEA, which was obtained in tests with meconium, shows that variants of CEA could be detected by our antisera. The specificity of these variants needs further study The PCA extracts of normal tissue had to be tested in protein concentrations from 40 to more than 165 mg/ml to detect CEA by our methods. In tumour extracts, on the other hand CEA is in most cases easily detectable in protein concentrations less than 1 mg/ml. The low concentration of CEA in normal tissue, and the variations seen in the CEA



Fig 5 Immunoelectrophoresis in agarose gel to show the association between the α- and β_E-protein.

Trough 23 and 26 Unabsorbed antisera nos. 23 and 26.

Well E Sephadex G 200 fraction (2. peak) of PCA extract of colonic carcinoma containing the β_E and α-protein.

Note the reaction of partial identity between the β_E and α-line, and the weak spur formed by the α-line The electrophoretic mobility of the α-protein is slightly slower than that of antitrypsin which precipitates in the same area. The most anodic line is precipitated albumin

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STUDIES ON *STACHYBOTRYS ALTERNANS*

III. Chromatographic separation and tissue culture toxicity test of stachybotrys toxins

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Chromatographic analyses and tissue culture toxicity tests were simultaneously carried out with stachybotrys crude toxin. Toxic fractions were localized by tissue culture toxicity tests, and further chemical analyses were concentrated on these parts. By using consecutive partition chromatography on silica gel columns with different solvent systems the crude toxin was resolved into three components which were toxic to mouse primary fibroblast culture. These three toxic stachybotrys components contained some impurities, they were non-fluorescent and they gave negative resorcinol test.

Many mycotoxins are mixtures containing several chemically and biologically different compounds. Thus e.g. the aflatoxins B_1, B_2, G_1, G_2, which have the same nature but differ somewhat in their chemical structures and biological effects, form a well-known mycotoxin complex. Ochratoxin is composed of ochratoxins A, B and C. In the present work chemical analytical methods and tissue culture toxicity tests were used in combination for separation and identification of the different stachybotrys toxins.

MATERIAL AND METHODS

Preparation of the Crude Toxin

The toxins were produced by cultivating *Stachybotrys alternans* (syn. *S. atra*, *S. chartarum*) strains either on the wheat-barley-oats (1 : 1 : 1) substrate or on modified Czapek medium. The chemically defined medium had the following composition:

NaNO	2.0 g
KCl	0.5 g
MgSO 7H_2O	0.5 g
FeSO 7H_2O	0.01 g
K_2HPO	1.0 g
ZnSO 7H_2O	0.01 g
CuSO 5H_2O	0.005 g
glucose	60.0 g
thiamine	0.5 µg/ml
nicotinic acid	0.025 µg/ml
ascorbic acid	0.3 µg/ml
distilled water ad	1000 ml

On both media selected *Stachybotrys* strains were cultivated 4–6 weeks at 22° C. The grain material was then dried 3–4 days at 55° C and the modified Czapek medium was homogenized and preserved freeze-dried. From these materials the toxic substance was separated by extraction with ether and precipitation with petroleum ether (*Korpinen & Uoti* 1974). The product is called crude toxin. The yield from the grain material consisted of a light-brown or dark-brown fine powder, its quantity varying greatly depending on the *Stachybotrys* strain used. The yield from the chemically defined medium was a dark-brown material.

Chromatography

The crude toxin preparations were fractionated, employing partition chromatography on silica gel (Kieselgel G, Merck) columns.

The following solvent systems were used:

acetone – benzene (1 : 1)
chloroform – petroleum ether – methanol
(20 : 19 : 1)

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Serum 60 with a staphylococcal antibody titre of 10000 (< 100000) was the only one reported to contain antibody

DISCUSSION

The separation of bound radiolabelled antigen from unbound radiolabelled antigen is of primary importance in all radioimmunoassays. In the test presented here, protein A containing *Staphylococcus aureus* was used for this purpose. Protein A combines specifically with the Fc-part of IgG types 1 2, and 4 which in normal sera comprises more than 90 per cent of the total IgG (9 18) Protein A containing staphylococci were first used by *Jonsson & Kronvall* in a RIA test for α-foetoprotein (7 8) and have also been used in a RIA test for the quantitation of insulin (4)

This solid phase system has several advantages. 1 The reaction between protein A and the combining site on the IgG molecule is almost instantaneous (10) 2. There seems to be no difference in the combining properties to protein A between free and antigen bound IgG 3 Radiolabelled antigen which has reacted with its specific antibody develops, after reaction with protein A fixed to the bacterium, into a solid phase which is very easily sedimentated by low speed centrifugation. It is not necessary before counting to wash the sediment after the supernatant containing the unbound radiolabelled antigen has been sucked off. These properties and the short incubation period necessary for the completion of the reaction between HBAg and HBAb, permit a fast assay Results to be obtained from single sera are ready in 2 hours.

By the purification procedures used for the HBAg, the use of the selected antisera and the incubation sequence of the assay the sensitivity level of the antigen competition test presented compared well with that of the Ausria test. The addition of labelled antigen to the reaction mixture after primary incubation of unknown serum and antiserum dose, increased the sensitivity about ten times as compared with that obtained by simultaneous mixing of all components (Fig. 4) It is likely that a higher sensitivity might be obtained if hyperimmunesera from guinea pigs were used. In the comparisons presented there was agreement in 38 out of 40 samples. Using the staphylococcal test, one serum was found to contain HBAb while the Ausria test was positive for HBAg In this case, it is likely that complexes were present. In the other specimen, the Ausria test gave a weak positive HBAg result in 75 per cent of the referee laboratories, while the staphylococcal test was negative. In a third specimen, No. 46, there was also a 75 per cent agreement between the referee laboratories, and the staphylococcal test was positive for HBAg

The specificity of the staphylococcal test for HBAg is high. If human convalescent antisera and the chosen test conditions including titration of HBAg in an undiluted human serum pool are used, specificity testings similar to those used in the Ausria are not necessary (13)

HBAb is in the staphylococcal test measured by direct binding of radiolabelled HBAg. The sensitivity is at least as high as that obtained by the indirect hemagglutination test. In series of sera from hepatitis patients during the course of illness and from a few persons in whom subclinical infection is discovered, seroconversion has been observed in all cases of HBAg positivity except in those where a HBAg carrier state developed. So far positive results including even low (1/1) titres, have not been neutralized (more than 100 sera) by the addition of 1/10 of the volume of a pool of normal human sera. This is considered an indication of the high purity of the HBAg preparation used and of a high specificity of the antibody test. By this test, the prevalence of HBAb has been found to increase with age among Norwegian blood donors (15) the prevalence being high among non-hospitalized young drug takers (1) and among Norwegian track finders (16)

To distinguish positive from negative results, the standard deviation from the mean is calculated, respectively for the antigen and

Thin layer chromatography (TLC) (Kieselgel G Merck, 0,5 mm) of the crude toxin was carried out with the following solvent systems

ethyl acetate – toluene (3 1)
acetone – benzene (1 1)
chloroform – petroleum ether – methanol (20 19:2)
chloroform – acetone – methanol (18 1 1)

The plates were developed up to 16 cm and examined in UV light (wavelength 365 nm)

Resorcinol Test

The resorcinol test previously used in the identification of stachybotrys toxins (Pályusik 1970) was carried out in test tubes for assaying the toxic fractions. On TLC the toxic bands were sprayed with resorcinol test reagent (0.1–0.5 per cent resorcinol in concentrated hydrochloric acid)

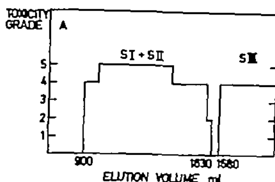
Biological Toxicity Test

Toxicity was tested in mouse primary fibroblast cultures as described by Ka praen & Ueli (1974) For the further steps in the chromatographic work it was often sufficient merely to find out which fraction or fractions were toxic. In some instances every fraction from the column chromatography but more often every second or every fifth fraction was tested for toxicity In the TLC the plate was cut into strips 1–2 cm wide and these were tested for toxicity The organic solvents were removed and the water extracts of the fractions used in the toxicity tests

After locating the toxic fractions by inoculation of undiluted material, crude quantitation of the toxin content was carried out when necessary for further steps in the chromatographic analysis. 10^{-} and 10^{-2} dilutions in distilled water were therefore tested in order to find the fractions with the highest toxin concentrations.

The cell cultures were examined 24 hours and if necessary 48 hours, after exposure to the test fractions. The toxic effect was assessed in the following scale toxicity 5 = all cells dead, 4 = nearly all cells dead, 3 = about half of the cells dead and microscopical changes in numerous cells, 2 = less than half of the cells dead and microscopical changes in many cells, 1 = slight changes in the cells. Those concentrations of the toxic substance which gave results within 24 hours and showed a toxicity of grade 4–5 were ideally suited for assessment and they were therefore regarded as optimum toxin concentrations.

For comparative purposes, BHK and U-cell lines were also tested for sensitivity to stachybotrys toxin with selected toxic fractions. Tests were run as with the mouse fibroblasts.



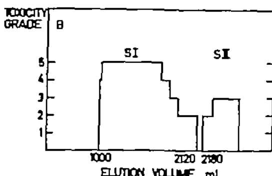


Fig 1 Chromatographic separation of stachybotrys toxin on silica gel columns. Toxicity tested with undiluted material.

A. Crude toxin eluted with acetone benzene column 4 × 95 cm, elution rate 90 ml/h.

B. Fraction SI + SII from crude toxin A) eluted with chloroform petroleum ether methanol column 4 × 90 cm, elution rate 100 ml/h

Fraction volumes 10 ml.

RESULTS

By using in two successive chromatographic columns the following solvent systems

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Fig 2 Effect of stachybotrys toxin on mouse primary fibroblast culture.

Control fibroblast culture, 48 hours old.

A) 100 × B) 294 ×

Fibroblast culture 24 hours after exposure to stachybotrys toxin. Stachybotrys toxin added to 24 hour old cell cultures.

Toxicity 5 grades.

C) 100 × D) 294 ×

May-Grünwald – Giemsa stain.

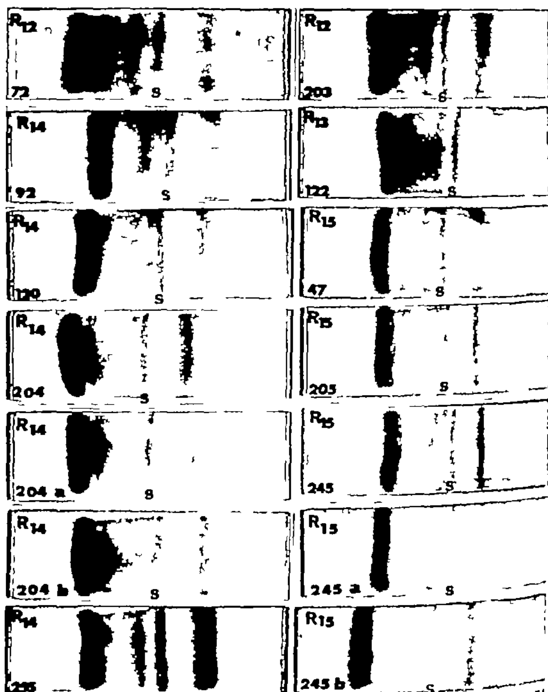


Fig 4 Electrophoreses of serum from rabbits immunized with *Klebsiella pneumoniae* type 2, strain T' and *Klebsiella pneumoniae* type 3 strain F 10.N Y (Table 3)

used. *Eichmann and co-workers* (2) suggested that the ability to form restricted antibodies was under genetic control. Neither the present work nor that by *Kimball et al* (9) are in support of this.

The pattern of restriction as well as the quantity of antibodies change continuously. A clone of antibody producing cells may proliferate for a period, to be replaced by another clone. At the same time two clo




B


C


D

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MULTIPLICATION OF *COWDRIA RUMINANTIUM* IN MONOLAYER OF TICK CELLS

M. P. Andreasen

C. ruminantium (*C.r.*) which gives rise to the disease heartwater in domestic ruminants and in some wild ruminants, was the first disease to be demonstrated as a rickettsiosis in animals (3).

Attempts to propagate *C.r.* *in vitro* have been carried out over many years. Initially the methods were based upon the same principles as those used for bacterial cultures. Later on, when tissue and cell cultures of mammalian cells had come into use and the *C.* organism finally was considered an obligate intracellular parasite, it was attempted to grow it in these cultures too. All attempts to propagate it *in vitro* have hitherto failed (4, 5).

As first described by *Cowdry* (2) the vectors of heartwater counting only a few spp. of the tick genus *Amblyomma*, do not only propagate *C.r.* in the epithelial cells lining their gut, but an additional observation by the author indicates that the vectors may also form an amplifier system, in qualitative sense, for the above organism. This observation, in particular was taken into account when the experiments described here were planned.

Nymphs of both *A. hebraeum* Koch 1844 and *A. variegatum* Fabricius 1794 were selected for preparation of the primary tick cell suspensions, at different stages of their metamorphosis. Prior to selection, the nymphs were fed on clinically healthy rabbits. All nymphs used in this work have been bred under strict control in a European laboratory in order to prevent undesirable contaminations of the tick strains. Their preceding stages were bred under the same conditions except that the adult stages were fed on scrotum of clinically healthy rams. The reproduction of the above ticks has been repeated throughout numerous generations in order to harvest populations of ticks which finally could be characterised as being "clean" and used as test material. The medium used for the primary cell cultures had the following composition: Hanks sol. with lactalbumin hydrolysate in which the pH was adjusted to values between 6.8 and 7.2 by addition of sodium bicarbonate. Just before suspension of the cells and seeding of these into culture flasks (Nunc Ltd.'s plastic flasks) the above medium was enriched with 10 per cent foetal bovine calf serum which prior to use had been heat inactivated for 1h at 56° C.

In some of the cultures, the medium was enriched with 5 per cent of an extract from tick eggs (1). Finally penicillin streptomycin and mycostatin in the following amounts were added: 50 i.u., 500 µg and 10 to 50 µg mycostatin per ml of final medium. At subcultivation of primary cells, these were grown in the above medium with the modification that no antibiotics or mycostatin were added. Sheep and lambs of European breeds, pure as well as mixed, served as experimental animals for infection with *C.r.* from inoculated tick cell monolayers. As control served a corresponding group of animals which received injections of a suspension of tick cells which prior to the injection had been exposed to *C.r.* negative blood. Finally groups of the above animals were used to determine whether the tick cell cultures per se were toxic to sheep if "clean" unexposed cell cultures were injected into the animals.

The applied strain of *C.r.* originated from an area in which the frequency of heartwater was high. The strain was given the name of this area Kibaha. Brain suspension from a cow which died from acute heartwater in this area was used for the isolation of the strain. Isolation was achieved by injection of this suspension into African sheep from this locality. At the stage of parasitaemia, infective blood from the latter was introduced by four serial passages in local breeds before infective blood from the last mentioned sheep could be used as the infective inoculum for the tick cell cultures.

The preparation in general of the tick cell cultures shall be described elsewhere (1). Subcultures of cells in plastic flasks, containing small cover slips, were inoculated on their 6th day of growth, when they usually formed an even monolayer. The medium was poured off the flasks, and the content

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1. acetone – benzene (1:1)
2. chloroform – petroleum ether – methanol (20:19:2)

the crude toxin was fractionated into three components which were highly toxic to fibroblasts. This result was reproducible. In Fig. 1 are given the details of the separation of these three components, denoted with S I, S II, S III. These components contained some pigment impurities.

TLC gave broad toxic areas with Rf values, varying in different solvent systems from 0.30–0.55 in chloroform-petroleum ether-methanol to 0.37–0.86 in benzene-acetone. The toxic fractions did not show any specific fluorescence in UV light.

As seen from Fig. 1, the majority of the toxic fractions, when tested in undiluted condition caused total destruction of cell cultures within 24 hours. The affected fibroblasts showed pyknosis, cytoplasmic vacuolation, fragmentation of nuclear chromatine and disappearance of the nuclear membrane. Fig. 2 illustrates the toxic effect on the cells.

BHK and U-cells proved less sensitive to the effect of the toxic fractions than mouse primary fibroblasts.

DISCUSSION

…e method of using chromatographic analyses and tissue culture toxicity test in combination was chosen as the most rapid and most reliable means for discovering the toxic fractions.

The employment of tissue cultures instead of experimental animals in the toxicity tests has several advantages. The economy in time and cost is self-evident. The toxin quantities obtained from the chromatographic analyses of the present study were often too small to be tested in experimental animals. The results of the toxicity tests in experimental animals are also sometimes difficult to interprete. The application of tissue cultivation in various mycotoxin studies has clearly increased in recent times (*Engelbrecht & Purchase* 1969, *Ohtsubo & Saito* 1970).

The primary cell culture technique is time consuming compared with the established cell line techniques. However, the mouse primary fibroblast culture was selected here instead of cell lines in view of its higher sensitivity. The sensitivity of the different cell and tissue cultures to various mycotoxins is known to vary greatly (*Cardeilhac et al.* 1972).

Bodon & Palyusik (1970) studied the effect of the toxic extracts from *Stachybotrys alternans* on primary calf kidney epithelial cells. The cell changes reported largely resemble those observed in the present study.

Three toxic components could be separated by the chromatographic procedure used. *Iushir* (1968a) has previously separated three fractions of stachybotrys toxins by TLC and alumina column chromatography. His fractions proved toxic when injected to mice, they were fluorescent and positive in the resorcinol test. In fact *Iushir* (1968b) has described a method for demonstration of toxic fraction based on the resorcinol test. The toxic fractions obtained here were found to be negative in the resorcinol test and they had no specific fluorescence. It is concluded that by the resorcinol and fluorescence tests components other than those demonstrated as toxic in the present test are measured. In addition, since the chromatographic methods and toxicity tests in *Iushir's* work differ from those applied here it cannot be concluded with certainty that the same toxic fractions would have been separated and studied. The three toxic components obtained by our method still contain some impurities.

The results obtained support the idea that *Stachybotrys alternans* produces three chemically different toxins. Further purification of the toxic fractions is needed before a more detailed chemical characterization is possible. Efforts in this direction as well as studies on biological properties of the fractions are in progress.

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treated with stachybotrys toxin. The main results emerging from the present experiments are statistically significant differences between the groups of treated and control animals in the frequencies indicated above.

The test animals, which were subjected to frequent control showed no definite clinical signs of any disease The mice in the groups fed with toxic grains during five days (Groups 6 13 14) appeared to lose some weight, however The average pregnancy percentage in all the control groups was 90.5 among the 8–9 animals of each group there was only one non-pregnant mouse or none The corresponding average for all those groups which received toxic material was 70 7 The difference is statistically significant. The differences in frequency of non-pregnant animals between the control mice and the three major toxin-treated groups (Table 2) are significant. An especially high proportion of non-pregnant animals was found in the groups in which the toxin was administered as a single dose on the third day of gestation (Groups 1 2) and in the two groups fed toxic grains during five days starting on day 5 and 10 respectively (Groups 13 14) The mycotoxin treated mice later found to be non-pregnant often showed indications that the animals had most probably been pregnant but had *aborted* In these animals the uterus was found to be abnormally large and the presence of corpus luteum in their ovaries confirmed that the ovulation had taken place

Dead and resorbed foetuses (Fig 1) were found in many groups. The foetus sizes were rather variable within several litters and the number of stunted foetuses was therefore recorded. As seen from Table 2, there are significant differences in the frequency of stunted, dead and resorbed foetuses between the control animals and the toxin-treated major groups (I II III) The percentage of such affected foetuses is highest in the major group III in which toxic feed had been given during various five-day periods of gestation the proportion decreased with the toxin dose administered, in the groups which received the toxin as a single dose. Subcutaneous haemorrhages were seen in a few foetuses both in the control and test groups

Statistically significant differences were further demonstrated as regards the average litter size of live normal foetuses between the combined group of control animals and the

TABLE 2. *Proportional Occurrence of Non-pregnant Females and of Normal and Affected Foetuses in the Major Groups of Mice Treated with Stachybotrys Toxin and Among Control Mice*

[illegible] jor group groups proper)	Per cent in the major group				
	Non-pregn. females	Normal foetuses	Stunted foetuses	Dead resorbed foetuses	Stunted, dead & resorbed foetuses
Controls (4 7 8, 9 15)	9.5	90.5	3.8	4.3	8.0
I (1 10 12)	33.3	60.0§	15.9§	11.2†	27.1§
II (2, 3)	33.3*	81 7*	9.5*	8 7	18 2†
III (6, 13 14)	32.0*	61.0§	25.7§	12 9§	38 6§

* = $p<0.05$.
† = $p<0.01$
§ = $p<0.001$
In the statistical treatments the χ^2 test was used.

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PHYLOGENETIC ASPECTS OF STAPHYLOCOCCAL PROTEIN A-REACTIVE SERUM GLOBULINS IN BIRDS AND MAMMALS

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Serum samples from 300 individual birds representing 72 species from 15 of 28 living orders were tested for protein A reactivity in gel diffusion experiments. Only two species, *Rhea americana* (Greater Rhea) and *Pterocnemia pennata* (Lesser Rhea), showed positive reactions, whereas serum samples from the remaining 70 species tested were negative. Protein A reactivity in the two Rheiformes species was identified in a slowly migrating serum globulin of unknown function with a molecular weight of 185 000. Absorption of serum from *Rhea americana* with staphylococci containing protein A almost completely eliminated the slow globulin. In *Pterocnemia pennata* similar absorption experiments reduced the amount of the corresponding globulin by 40–50 per cent. Two mammalian sera, the wooly opossum and the wallaby, not tested previously were also analyzed. Both species showed marked protein A reactivity. The only negative mammalian serum found earlier, the American opossum, was also reexamined. Concentrated globulin fractions showed a quantitatively small but definite reactivity in tests for protein A reactivity. Thus, all 65 mammalian species tested contain gamma globulins reactive with protein A presumably via similar Fc structures of immunoglobulin G with a common evolutionary origin.

Protein A from *Staphylococcus aureus* shows unique reactivity with the Fc part of human γ-globulin (5, 10). This reactivity correlates with IgG subgroups, IgG-1, 2 and 4 globulins being protein A reactive (13). A similar Fc reactivity has also been shown for rabbit γG-globulin (6), guinea pig γ_1- and γ_2-globulin (3, 4) as well as for mouse γ_{2a}, γ_{2b}, γ_1-globulin (11). In a previous report results from studies of protein A reactivity of serum samples from various animal species indicated that Fc mediated protein A reactivity is a characteristic of gamma globulins in most mammalian species (12). The current investigation provides further evidence supporting the hypothesis for a common phylogenetic origin of mammalian protein A reactivity.

One notable exception to the negative pattern among non mammalian orders of animals found in our previous studies was encountered in serum from the Greater Rhea, *Rhea americana*, which was strongly positive in tests for protein A reactivity (12). We have now extended our studies among birds to cover 300 serum specimens. *Only two*

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Fig 1 Three pregnant uteri of experimental Group 12. Arrows point to resorbed foetuses. Fresh, unstained specimens.

major toxin-treated groups (Table 2) In the test groups proper the average litter sizes were regularly smaller than in the control groups proper with the exception of Group 5 the animals of which had received a very low dose of toxin early in gestation.

Between the five different control groups no statistically significant differences were found in the frequency of non-pregnant animals nor in that of affected foetuses. On this basis the lumping of the control groups for the purpose of statistical treatment was considered justified.

Histopathological Studies

No abnormalities were observed in the control mice, except slight infiltrations and foci by lymphocytic cells in the liver Haemosiderin was abundantly present in the uterine tissues of the non pregnant animals which received toxin (Fig 2) In the uterine wall of the pregnant animals of this group hyperaemia, haemorrhages and subepithelial oedema were noted (Fig. 3) The foetal membranes were hyperaemic with haemorrhages and blood was present between the uterine wall and the foetal membranes, too. No structural changes were noted in the foetuses. The liver and the kidney of the control and that of the toxin treated mouse appeared similar

DISCUSSION

The results of this study confirmed the working hypothesis that stachybotrys toxin has a detrimental effect on the pregnancy of mice. This effect can be accomplished by doses low enough to cause no other definite clinical signs of disease in the mothers. The evidence is offered by the statistically significant differences found in the frequency of non-pregnant mice, in the average number of live foetuses per animal and in the differences of percentages of affected foetuses between the groups treated with toxin and the control groups.

The average pregnancy percentage of 90.5 observed in the control groups of this study corresponds with the level usually obtained by this method of pregnancy determination (Rugh 1968) The definitely higher than normal frequency of non-pregnant animals in the test groups is considered to be caused by the stachybotrys toxin. The state of non-pregnancy when the result of toxin effect, has with all likelihood developed either by prevention of implantation or by abortion. The low number of pregnant animals in the groups (Groups 1 2) in which the toxin was administered on the third day would seem to suggest that stachybotrys toxin rather effectively prevented the implantation (which normally takes place on the day 4½ of gestation) in those groups. The absence of non-pregnancies among the mice of Group 6 does not favour this explanation. Prevention of implantation gives no explanation for the

Rheiformes birds were positive whereas the remaining 70 species tested were all negative.

MATERIALS AND METHODS

Animal sera. Blood samples were obtained from birds caught at ornithological field stations in Sweden and from birds and mammals in various zoological gardens in USA and Denmark. Serum from *Pterocnemia pennata* was kindly supplied by Dr *R. Faust* Zoologischer Garten, Frankfurt am Main West Germany Cassowary and Jungle fowl sera were a gift from Dr *Albert A. Benedict* Honolulu Hawaii Serum samples were stored at −20° C until use.

Determination of Protein A Reactivity

Testing for protein A reactivity was performed in agarose gel diffusion experiments as described previously (13) Protein A was purified from *Staphylococcus aureus* strain Cowan I according to *Jensen* (7) or using immunosorbent techniques (8) The protein A precipitating myeloma globulin JLO (IgG-1 kappa) used in the test was isolated by zone electrophoresis on starch block (14)

Antisera Against Rhea Serum Proteins

Whole serum from *Rhea americana* and *Pterocnemia pennata* as well as fractions isolated by block electrophoresis were emulsified with Freund' complete adjuvant (Difco) and injected into rabbits Booster doses were given after one month and the rabbits bled weekly thereafter Selected antisera obtained in this way were used throughout the studies.

Absorption Experiments

Staphylococcus aureus strain Cowan I and Wood 46, were grown in CCY broth (2) or on the same medium solidified with 1.5 per cent agar Both live bacteria and bacteria stabilized with formaldehyde and heat (9) were used in absorption experiments. To one volume of packed bacteria an equal volume of serum was added. After thorough stirring the bacteria were spun down and the clear supernatant frozen until further analyses.

Immunoelectrophoretic Analysis

Immunoelectrophoretic analyses of serum or serum fractions was performed using Gelman equipment. Crossed immunoelectrophoresis according to Laurell was also utilized giving better separation of serum fractions and permitting semiquantitation by means of planimetry (15)

Separation of Serum Proteins from Birds

Serum samples from Rheiformes birds were separated by block electrophoresis in agarose (15) or by gel filtration on Sephadex G-200 and G-150. Agarose blocks containing separated serum and measuring 180 × 95 × 4 mm were cut in slices 5 mm wide which were then frozen in test tubes. Fractions obtained after thawing of these slices and subjected to repeated extractions were analysed for protein content and protein A reactivity Sephadex G-200 (column diam. 50 mm, height 900 mm) was equilibrated with 1.0 M NaCl, 0.1 M Tris pH 8.0 at 4° C. The eluate was collected in 10 ml fractions at a rate of 30 ml per hour Fractions were read at 280 mμ. Concentration of pooled fractions was achieved by pressure filtration using Amicon equipment or collodion bags. In some experiments Sephadex G-150 was used. For estimations of molecular weights the columns were calibrated using human serum albumin and human IgG-globulin.

Protein Determinations

Protein curves on column eluates were obtained by measuring O.D. at 280 mμ. In other instances the protein concentration was determined by a modified Folin method (18)

RESULTS

Protein A Reactivity in Birds

In our previous study serum from one of seven bird species was strongly positive in tests for protein A reactivity (12) In order to cover the whole spectrum of birds more completely we extended our studies to include 300 bird serum specimens from 72 different species. These birds represent 15 of 28 living orders including most primitive orders. Of all the sera tested only those from the two Rheiformes species, *Rhea americana* and *Pterocnemia pennata* showed protein A reactivity (Table 1) Eight serum samples from five individual Rheiformes birds were similar in their inhibitory activity in the test system. The magnitude of this protein A reactivity was of the same high level as the one detected in human or rabbit serum. The concentrations of protein A reacting gamma globulins in normal serum from the two mammalians amount to as much as about 1 g per cent. Serum samples from the remaining 70 bird species studied were all protein A negative



Fig 2 Light micrograph of the uterine wall of a non-pregnant mouse of Group 12, showing accumulations of haemosiderin in macrophages (arrows) HE, × 540.

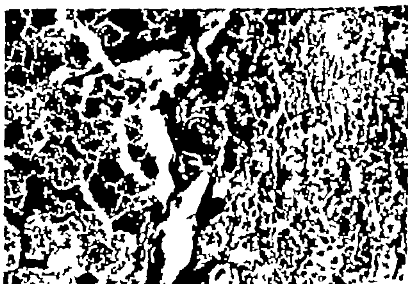


Fig 3 A pregnant mouse of experimental Group 12. Haemorrhages are seen in foetal membranes, and the uterine wall is oedematic. HE, × 318.

high number of non-pregnant animals in Groups 13 and 14 in which toxic feed was given during days 5–9 and 10–14 respectively The larger than normal uteri of the non pregnant mice in the said groups support the assumption of abortion as the likely cause of non-pregnancy That the two non-pregnant mice of group 12 had aborted is also indicated by the results of histopathological studies haemosiderin was detected, constituting a sign of bleedings in the uterine wall.

The statistically significant differences in

TABLE I Protein A Reactivity of Serum Samples from 72 Bird Species. The Individual Species Tested Are Indicated in the First Six Orders Only

Order	Species	Protein A reactivity
Sphenisciformes	Humboldt Penguin (*Spheniscus humboldti*)	–
Struthioniformes	Ostrich (*Struthio camelus*)	–
Rheiformes	Greater Rhea (*Rhea americana*)	+
	Lesser Rhea (*Pterocnemia pennata*)	+
Casuariiformes	Cassowary (*Casuarius casuarius*)	–
	Emu (*Dromaeus novae-hollandiae*)	–
Tinamiformes	Great Tinamou (*Tinamus major*)	–
Pelecaniformes	American White Pelican (*Pelecanus erythrorhynchos*)	–
	White Pelican (*P. onocrotalus*)	–
	Brown Pelican (*P. occidentalis*)	–
	Dalmatian Pelican (*P. crispus*)	–
	Australian Pelican (*P. conspicillatus*)	–
Anseriformes	(4 species)	–
Falconiformes	(2 species)	–
Galliformes	(5 species)	–
Charadriiformes	(13 species)	–
Columbiformes	(2 species)	–
Psittaciformes	(1 species)	–
Strigiformes	(1 species)	–
Piciformes	(2 species)	–
Passeriformes	(32 species)	–

Thus occurrence of protein A reactive serum proteins appeared to be very unusual among birds, being documented only among Rheiformes.

Protein A Reactive Serum Globulins in Rheiformes Birds

Protein A reactivity detected in the two Rheiformes birds, *Rhea americana* and *Pterocnemia pennata* was studied further by absorptions of serum samples with the protein A containing *Staphylococcus aureus* strain Cowan I. The absorbed sera were analysed by immunoelectrophoresis as well as by crossed immunoelectrophoresis. Serum samples absorbed with the Wood 46 strain of *Staphylococcus aureus* lacking protein A were used as controls. As shown in Fig. 1 A, absorption of serum from *Rhea americana* with Cowan I resulted in complete disappearance of a precipitation line in the slowly migrating globulin region. Another precipitation line of similar but more restricted electrophoretic mobility was unchanged. Cowan I absorption of serum from *Pterocnemia pennata* gave similar but not as dramatic results (Fig. 1 B) with a reduction apparent in a slow globulin fraction. Absorbed serum samples subjected to crossed immunoelectrophoresis showed a substantial decrease of the slow globulin peak in Cowan I absorbed serum from *Pterocnemia pennata*. Planimetric analysis indicated a 40–50 per cent reduction.

Sera absorbed with Cowan I also showed another change as compared to the control sera absorbed with Wood 46 or unabsorbed sera. This change was most apparent in *Pterocnemia pennata*. A β-globulin located closer to the application well was almost completely absent following such absorption.

Protein A reacting serum globulins in Rheiformes birds were also identified using another approach. Rabbit antiserum to whole serum from *Rhea americana* was neutralized with a) Cowan I-absorbed serum from *Rhea americana* and b) Wood 46-absorbed serum from the same bird. Antiserum neutralized





Fig 1 Rabbit skin test. The reaction, of 3 days old, caused by stachybotrys preparation No. 71 applied (at 0.05 ml) on the skin of a rabbit. From left to right. 10^{-4} 10^{-3} 10^{-2} 10^{-1} dilutions, acetone control.

Fig 2 Brine shrimp, *Artemia salina*, native preparation, × 40.

with *Michael et al.* (1956) The results were read after 2 days incubation. The titre was determined as the highest dilution which caused at least 80–90 per cent of the larvae to sink.

The titration of every preparation was repeated 3–4 times.

RESULTS

Table 1 gives the results of the toxicity measurements obtained by the three different methods. The order of sensitivity as regards most preparations tested turned out to be the mouse fibroblast test most sensitive, the brine shrimp test least sensitive. A preparation-dependent variation in relative sensitivity between the rabbit skin test and the mouse fibroblast test was revealed. The sensitivity of the latter ranged from 2 to 80 times higher. In case of one preparation (S I) the sensitivity of the rabbit skin test was 2.5 times higher than the sensitivity of the mouse fibroblast test. The preparation S III showed a low degree of toxicity. When the toxicity of S III for mouse fibroblast was concentrated 15 times, the concentrate still remained negative in the rabbit skin and brine shrimp test.

In the brine shrimp tests only the results from titrations by tenfold dilution were taken into account because of the difficulties in end-point determination from the series of twofold dilutions of toxin.

In the measurement of toxicity of verrucarin A and roridin A the same order of sensitivity of the tests as for stachybotrys toxin preparations in general was demonstrated. The mouse fibroblast test detected a level of 0.1 µg/ml verrucarin A and 0.05 µg/ml roridin A. In the rabbit skin test the figures were 1 µg/ml and 0.25 µg/ml and in the brine shrimp test, 10 µg/ml and 10 µg/ml, respectively.

TABLE 1 *Toxicity of Various Stachybotrys Toxin Preparations as Measured by Three Different Tests*

Toxin preparation	Toxin titre */0.1 ml		
	Rabbit skin test	Mouse fibroblast test	Brine shrimp test†
3,6	1600	4000	10
62	400	800	10
71	80	2000	10
72	400	800	
S I	1600	640	10
S II	40	3200	10
S III	0	4	0

*Reciprocal of the degree of dilution.
†Tenfold dilutions only tested.

DISCUSSION

The three methods applied in the present study showed clear differences in sensitivity. It can be concluded that most often the mouse fibroblast test proved superior in sensitivity to the rabbit skin test and that the

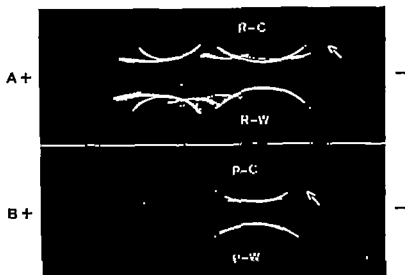


Fig. 1 Immunoelectrophoresis of *Rhea* serum samples absorbed with staphylococci with or without protein A. Fig. 1 A. Serum from *Rhea americana* absorbed with the protein A rich Cowan I strain (well marked R-C) and the Wood 46 strain not containing protein A (well marked R-W). Fig. 1 B. Serum from *Pterocnemia pennata* absorbed with Cowan I (well marked P-C) and with Wood 46 (well marked P-W). The rabbit antiserum used was raised against *Rhea americana* serum. Arrows indicate protein A reactive serum globulins.

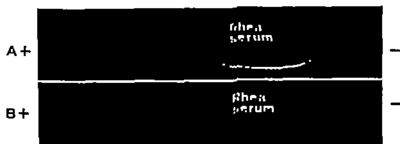


Fig. 2 Immunoelectrophoresis of serum of *Rhea americana* using neutralized antisera. Fig. 2 A. The rabbit anti-*Rhea* serum was neutralized with *Rhea* serum absorbed with *Staphylococcus aureus* strain Cowan I. Fig. 2 B. The rabbit anti-*Rhea* serum used was neutralized with *Rhea* serum absorbed with *S. aureus* strain Wood 46.

according to a) should still contain antibodies against protein A reacting serum globulins. Immunoelectrophoretic analysis of normal serum from *Rhea americana* using the two neutralized antisera is shown in Fig. 2. Both the slow gamma globulin as well as the β globulin were clearly demonstrated. A third line was present in the test samples as well as in the control experiments and could therefore be attributed to insufficient neutralization of antibodies directed against a minor serum component.

Serum from *Rhea americana* was subjected to gel filtration on a Sephadex G-200 column in order to further characterize protein A reacting globulins. The elution profile obtained showed three main peaks with a rather marked macroglobulin fraction (peak I). The slowly migrating globulin previously shown to react with protein A was detected

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THE ULTRASTRUCTURE OF CULTIVABLE TREPONEMES

2 Treponema calligyrum, Treponema minutum and Treponema microdentium

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Organisms of the species *Treponema calligyrum*, *Treponema minutum* and *Treponema microdentium* were studied in the electron microscope after negative staining. Statistical analysis demonstrated a difference in the distribution of cell lengths and in mean wave lengths between organisms of the three species. The cells of all species were covered by a regularly structured surface layer. The substructure of these layers showed variation from species to species but was identical for four strains of one species (*T. microdentium*). Generally individual cells of the species possessed two-three flagella inserted at each end. The flagella were entwined around the cytoplasmic body of the treponeme and individual flagella interdigitated in the mid-region. Treatment of cells with sodium deoxycholate or *Myxobacter AL-1* protease I revealed bundles of cytoplasmic tubules in the cell interior. It is concluded that the four strains of *T. microdentium* examined all belong to one and the same species, and that the morphological differences observed between treponemes of the three different species justify the maintaining of these as separate species.

This paper presents the second part of a comparative study on the ultrastructure of different species of *Treponema* of which the first part was completed recently (2). The ultrastructure of different species of treponemes was studied by identical preparative methods in order to disclose morphological characteristics of possible taxonomic significance. From previous electron microscopical investigations on cells of strains of *T. calligyrum*, *T. minutum* and *T. microdentium* (1, 4, 5, 6, 7) conclusions of taxonomic value cannot be drawn, mainly due to the various methods of preparation for electron microscopy applied by the investigators.

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In the main, the present study is confined to one strain of each species. Several independently isolated strains of *T. microdentium* are available (8), however, and since preliminary studies revealed morphological differences between organisms of the three species mentioned, it was found worthwhile to include different strains of this last species in order to ascertain whether or not these exhibited morphological variations.

MATERIAL AND METHODS

The strains of *T. calligyrum* and *T. minutum* labelled CIP 6441 and CIP 5162, respectively, were received from Dr. *P. Thibault*, La Collection de l'Institut Pasteur, Paris, France, and had, according to *Mouton* & *Gratton* (6), originally been isolated from genital lesions by Dr. *R. Vincent* in 1934.

Three strains of *T. microdentium* labelled CIP

in peak II (Mw ~ 185,000) The protein A reactivity in the eluates was confined to this second peak. The other slow globulin with a more restricted electrophoretic mobility and without protein A reactivity was found in peak III showing an approximate molecular weight of 75 000 Separation of serum from *Pterocnemia pennata* on Sephadex G-150 showed similar results and confirmed the molecular weight of the main protein A reacting globulin.

Protein A Reactivity in Mammals

Serum samples from two marsupial species not studied previously the Ecuadorian Woolly Opossum *Caluromys lanatus* and the Red necked Wallaby *Wallabia rufogrisea* were tested for protein A reactivity Serum from both species showed marked inhibition indicating presence of structures capable of combining with protein A. This positive reaction noted for the woolly opossum makes the previous negative result obtained with serum from a closely related species, the American opossum *Didelphis marsupialis* seem rather unexpected (12) Since the test system used does not detect protein A reacting gamma globulin at concentrations below approximately 40 mg per cent (10) it was felt that low concentrations of protein A reacting gamma globulins might still be present in serum of the American opossum. In addition, agarose electrophoretic analysis of serum from this marsupial showed a marked decrease of slowly migrating gamma globulins in contrast to serum from other animals tested Serum from the American opossum was therefore brought to 50 per cent saturation of ammonium sulphate the precipitate formed was washed twice and finally dissolved in one fifth of the original volume. This concentrated material showed slight but definite inhibition in tests for protein A reactivity

DISCUSSION

The finding of staphylococcal protein A reactivity in serum from the primitive South American bird *Rhea americana* (Greater Rhea) raised the question as to whether protein A reactivity is inherited in specific immunoglobulins similar to the protein A reactivity of mammals localized to the stable Fc part of immunoglobulin G and not the result of antigenic stimulation (12) Seven serum samples from four individual birds have now been tested in addition to a serum sample from the other Rheiformes bird, *Pterocnemia pennata* (Lesser Rhea) All of them showed a similar high degree of inhibition in tests for protein A reactivity The serum reactivity was further localized to an electrophoretically heterogeneous globulin with a molecular weight of 185,000. This slowly migrating globulin in serum from *Rhea americana* was almost completely removed by the protein A containing strain of *S aureus* Cowan I Similar absorption resulted in a 40–50 per cent decrease in the concentration of corresponding globulin in serum from *Pterocnemia pennata* The similarities in molecular weight and electrophoretic heterogeneity as well as mobility of the gamma globulins with data on chicken immunoglobulin Y would suggest that the gamma globulins in question might be analogous immunoglobulins (16 23) The presence of protein A reactivity in most of these molecules in all seven serum samples from *Rhea americana* indicates that the reactive structures are probably inherited in stable parts of the molecules and the activity thus is not the result of an antibody-activity It remains, however to determine the possible antibody nature of the globulins as well as the molecular localization of the protein A reactivity

It is now clear that all sera from 65 mammalian species tested contain proteins capable of reacting with protein A. This reactivity has been located to the Fc fragment of human (5 10) rabbit (6) guinea pig (3 4) as well as mouse gamma globulin (11) The reactivity found in all other mammals therefore most likely represents a similar Fc located reactivity Further proof for this generalization has recently been provided by *Lind et al* (17) After absorption of serum from man, dog, swine cow and sheep with the protein A

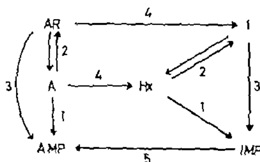


Fig 1 Possible known routes for conversion of adenine to AMP 1-adenine (A) and 1-hypoxanthine (Hx) phosphoribosyltransferase (EC 2.4.2.7 and EC 2.4.2.8) 2-purine-nucleoside phosphorylase (EC 2.4.2.1) 3-adenosine (AR) and 5'-inosine (I) kinase (EC 2.7.1.20 and EC 2.7.1.73) 4-adenine and 4'-adenosine deaminase (EC 3.5.4.2 and EC 3.5.4.4) 5-adenylosuccinate synthetase and lyase (EC 6.3.4.4 and EC 4.3.2.2) (4)

inosine 5'-monophosphate (IMP) and from this to AMP (Fig 1) (29)

III Adenine deaminase, EC 3.5.4.2 (4)
adenine + H_2O = hypoxanthine + NH

Hypoxanthine may be converted to IMP by hypoxanthine phosphoribosyltransferase or via inosine to IMP (Fig. 1) (29)

MATERIALS AND METHODS

Strains. The meningococci were the wild-type strain M1 of group B and the auxotrophic mutant 6-1b of strain M1 The mutant strain which requires histidine, proline, adenine and guanine for growth (*his pro A G*) was isolated by selective procedure with methotrexate from the mutant M1 *his pro* (15, 19) An *Escherichia coli* K12 strain was used for comparison in some of the experiments (17 19)

Media. Blood agar plates or basal medium (Medium A) plates were used as solid media (15) The basal medium plates were supplemented with the growth factors required (27) Fluid medium was the enriched, defined Medium KC (19) Fluid medium for E. coli was that of *Davis & Mingioli* (5)

Growth conditions. N meningitidis was grown as previously described (15 19) *E. coli* was suspended in the fluid medium, grown overnight at 37° C with shaking and diluted into fresh prewarmed medium the next day and grown to the desired density before use.

TABLE 1 *Reference Values of Standards Used for Identification of Radioactive Metabolites in Ethanolic Extracts from Whole Cells of N meningitidis and E. coli*

Internal standard	$R_{adenine}$ Isobutyric acid phase*	n-Butanol phase**
Adenine	1.00	1.00
Hypoxanthine	0.52	0.81
Guanine	0.61	0.75
Xanthine	0.42	0.74
Adenosine	0.82	0.87
Inosine	0.50	0.71
Guanosine	0.51	0.67
Xanthosine	0.20	0.58
AMP	0.37	0.40
ADP	0.21	0.19
ATP	0.12	0.11
S-AMP	0.06	0.11
AICA-R	0.28	0.25
IMP	0.09	0.21
IDP	0.04	0.10
ITP	0.02	0.04
GMP	0.07	0.16
GDP	0.03	0.08
GTP	0.02	0.05
XMP	0.05	0.15
XDP	0.02	0.07
XTP	0.01	0.03

Abbreviations S-AMP = adenylosuccinate, AICA-R = 5-aminoimidazole-4-carboxamide ribonucleotide. * Isobutyric acid phase isobutyric acid-conc. NH H_2O-0.1 M EDTA (500.21:279 8, by vol.) ** n-Butanol phase n-butanol-acetone-acetic acid-conc NH, H_2O (45 15 10 2.28, by vol.) (17) Before the run in this phase the paper was impregnated with the isobutyric acid solvent and dried.

Cell free extracts. Unless otherwise stated, extracts were prepared as in previous works (17 18) In most experiments they were used immediately Protein was estimated by the procedure of *Lowry et al.* with bovine albumine as standard (28)

Assay for labelled intermediates in intact cells. The procedure was essentially as before (17) One ml culture with approximately 1.5 × 10⁹ colony-forming units (C.f.u.) was mixed with 2.2 μg ¹⁴C-8-adenine (60 μCi/μmole) and run for 15 60 and 120 seconds. The cells were washed three times with 1 ml ice-cold KC medium, or with that used by *Davis & Mingioli* (5) Extraction of the labelled intermediates was performed by a method similar to that described by *Smith-Kielland* (33) The cells were heated with one ml aqueous 50 per cent ethanol for 6 minutes at 100° C. After cen-

containing staphylococcal strain Cowan I the slowly migrating gamma globulins were almost completely removed. Protein A reactivity could therefore be attributed to certain classes of gamma globulin in these species. The reactive, slow gamma globulin in the cow and the sheep made up only a minor portion of the total immunoglobulin concentration. This is in good agreement with earlier data showing only weak reactivity in these animals as well as in other artiodactylae (12).

The immune response of the American opossum has been extensively studied by others. *Rowlands Jr* & *Dudley* (21) and later *Taylor* & *Burrell* (22) documented the presence of both 19 S and 7 S immunoglobulins. The switch to 7 S production was delayed, however as compared to other mammals (20). *Marx et al.* (19) have shown a defective central as well as efferent limb in the immune system of the American opossum. These data are in good agreement with our findings of a barely detectable protein A reactivity combined with very low amounts of slow gamma globulins as revealed by electrophoresis.

The origin of the gamma globulin Fc structure capable of reacting with staphylococcal protein A and shown to be common to all mammals and not present in birds must have occurred after their estimated last common ancestry 285 million years ago. Recent analysis of amino acid sequences of myoglobulin and hemoglobulin has provided a date for the kangaroo-eutherian divergence (1). Since this was estimated to have occurred about 138 million years ago protein A reactive immunoglobulin structures studied can be dated to at least this age. Its presence in the echidna, a monotreme, would place the date much earlier, to perhaps 200 million years ago. Thus using protein A gamma globulin reactivity as marker for structural features may provide additional insight into the evolution of immunoglobulin structure.

The excellent technical assistance of Mrs. *Marian* *e Waldheim* and Miss *Kerst* *Lu dberg* nd the secretarial expertise of Mrs. *Edi Schönnandt*, Mrs. *Britt-Marie Karlsson* and Miss *Shitaye Welde Kidane* is gratefully acknowledged.

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N meningitidis when cells are grown in the presence of radioactive adenine (16) is then the result of the activity of this enzyme since no other metabolic route seems to lead from adenine to its ribonucleotides in *N meningitidis*.

N meningitidis seems to lack enzymes for conversion of adenine to adenosine (Table 7). The enzyme purine-nucleoside phosphorylase (PNPase) which is found in a variety of microorganisms readily catalyzes reversible reactions between purine bases and R 1 P (32). A synthesis of adenosine from adenine and R 1 P could not be demonstrated by extracts from *N meningitidis* in contrast to extracts from *E. coli* (Table 7), not even by extracts containing dithiothreitol for protection of possible sulphhydryl groups (32). No PNPase activity could be induced in *N meningitidis* by addition of adenosine as otherwise found in *E coli* (Table 7) (6). Spectrophotometric assay of PNPase with inosine and xanthine oxidase (6, 20) revealed no activity of PNPase in *N meningitidis* extracts in contrast to extracts from *E. coli* (*S Jyssum* to be published). The hydrolytic enzyme adenosine nucleosidase seems not to catalyze the synthesis of adenosine from adenine and ribose (30).

Adenine deaminase could not be demonstrated in extracts from *N meningitidis*. The enzyme is found in microorganisms such as *Azotobacter vinelandii* (8), *Candida utilis* (7) and *Schizosaccharomyces pombe* (1). The enterobacteria *E. coli* and *Salmonella typhimurium* are reported not to have this enzyme (12). The absence of this enzyme in *N meningitidis* is consistent with the finding that the adenine-guanine mutant cannot grow on adenine as the only purine, but grows well on hypoxanthine alone (Table 2).

The extracts from *N meningitidis* had pronounced activities corresponding to adenylate and adenosinediphosphate kinases (Fig. 11). Since AMP cannot support growth in combination with guanine of the adenine-guanine mutant (Table 2) it may be assumed that intact AMP is not taken up by *N meningitidis* cells, and AMP is not broken down to adenosine or adenine outside the cell.

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TABLE 1 *Comparison Between Optical Density (A_{550}) and Dry Weight (mg/ml) of Cells of Different Phases of Growth*

Growth phase	Optical density A_{550}	Dry weight mg/ml	$\frac{A_{550}}{\text{mg/ml}}$
Log.	4.32	1.35	3.20
Log.	6.18	1.90	3.25
Log.	6.60	2.00	3.30
Log. Lyt.	6.96	2.20	3 17
Lyt.	5.83	1 75	3.33
Lyt.	2.90	0.90	3.22

Log. = logarithmic growth phase.
Lyt. = autolytic phase.

from cultures growing in the fermentor. The samples were centrifuged, the cells were washed once and then resuspended in 32 ml a.d. The centrifugation of the samples, the washing and resuspension procedures were made according to the method described above. Two ml were taken from this suspension and after a 100-fold dilution, the optical density was determined as before at 550 nm. The remaining 30 ml of cell suspension was centrifuged at 20,000 × g for 10 min and the pellet was dried at 105 C for 18 hours and weighed to the nearest 0 01 mg. The relation between A_{550} readings and the dry weights of the cells of different phases of growth are recorded in Table 1 No significant variations in these quotients occurred. The optical density of cell suspensions of this strain, prepared according to our method, could thus be used as an estimate of bacterial concentration in both logarithmic and a tolytic phases of the growth cycle.

RESULTS

Preliminary experiments were performed to compare the effects of two peptone media on the yield of hyaluronidase. Series of 50-ml flasks containing media of the following composition protease peptone and trypticase totally 40 g/l in varying proportions and glucose, salts, vitamins and trace elements as in the preculture medium were inoculated simultaneously with cells from the same suspension and incubated for 15 hours at 37 C. The hyaluronidase activity of the culture supernatants increased with the proteose peptone content of the media and was close to zero in the medium containing trypticase only No definite conclusions regarding the effect of variations in the medium composition on the formation and release of hyaluronidase from these experiments could be drawn since the final pH of the media varied. However on the basis of these experiments, proteose peptone was used as the nitrogen source in this investigation.

The growth cycle From the growth curves of Figs. 1 and 2 it can be concluded that glucose was limiting the final bacterial yield and that the exponential growth phase turned directly into a phase of autolysis upon the exhaustion of glucose. The amount of titrant used to keep pH constant is plotted against time in Fig 2. Cell turbidity and the amount

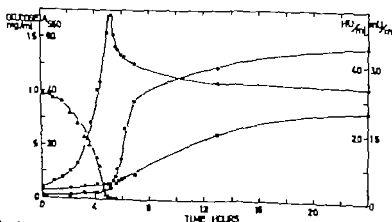


Fig 1 Relationship between bacterial concentration, glucose concentration and enzyme activities in the culture medium. Bacterial concentration (A_{550}) ○——○, glucose concentration (mg/ml) ▲——▲, hyaluronidase activity (H.U./ml) ●——● aminopeptidase activity (mU/ml) □——□.

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CAPACITY OF GROUP A, B, C, D AND G STREPTOCOCCI TO AGGLUTINATE SENSITIZED SHEEP RED CELLS

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The reactivity of streptococci with sheep red cells, sensitized with a subagglutinating dose of rabbit anti-sheep red cell antibodies was studied agglutination of sensitized and not non-sensitized sheep red cells by streptococci was demonstrated in isolates belonging to groups A, B C, D and G The frequencies of isolates which had the capacity to agglutinate were 97 88 and 100 per cent in group A, C and G but only 47 and 33 in group B and D The agglutination of sensitized sheep red cells by streptococci diminished after treatment of the streptococci with protease from *Streptomyces griseus* but was not affected by pepsin or trypsin treatment. Heating the streptococci at 60° C for 30 minutes and repeated subculturing did not suppress their agglutinating activity Besides, various streptococcal extracts agglutinated sensitized sheep red cells. The presence of an immunoglobulin G reactivity in streptococci might cause certain difficulties in the serological grouping and typing of streptococci.

An interaction between streptococci groups A, C and G and gammaglobulin was recently described (12) The gammaglobulin structures involved were confined to the IgG class they were not demonstrable in IgA, IgM IgD or IgE. Only intact immunglobulin not F(ab) or Fc preparations of immunglobulin G reacted. By means of the interaction, streptococci agglutinate sheep red cells, sensitized with a subagglutinating dose of rabbit anti sheep red cell antibodies.

In the present investigations the experiments were designed to study the capacity of streptococci and streptococcal extracts covering different serological groups, to agglutinate sensitized sheep red cells. Some properties of the streptococcal factor responsible for the agglutination of sensitized sheep red cells were also elucidated.

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MATERIALS AND METHODS

Streptococcal Strains

The streptococci were isolated from throat, ear urine bronchial secretions, wounds, vagina, nose and urethra at the Department of Clinical Microbiology University Hospital, Lund, Sweden. In all, 268 isolates were studied. Blood agar plates were inoculated and incubated over night. Streptococci were subcultured anaerobically on blood agar and afterwards in Todd-Hewitt Broth (Sigma) and frozen at −21 C until subcultured further.

Serological Grouping of Streptococci

Grouping of the streptococci was performed as described previously (2) by use of specific rabbit antibodies to Lancefield groups A, B, C D and G

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BIOCHEMICAL STUDY OF PROTEUS INCONSTANS (PROVIDENCIA)

Occurrence of Urease Positive Strains

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The biochemical reactions of 8 isolates of *Proteus inconstans* biotype A, 83 isolates of *P inconstans* biotype B and 18 isolates of *P rettgeri* were compared with 14 isolates that had the same biochemical pattern as biotype B of *P inconstans* but for a positive urease reaction. On the basis of comparison it is shown that the atypical isolates must be considered as urease positive variants of *P inconstans* biotype B. The urease reaction is accordingly not an absolutely reliable feature in differentiating *P inconstans* from the other *Proteus* species. This confirms the observations of some previous authors but contradicts the more generally accepted view. Attention is drawn to the possible value of a negative galactose fermentation test in characterizing strains of *P inconstans* biotype A.

Over a period of two years rather more than 300 strains of *Proteus inconstans* were isolated in the laboratory mainly from urine specimens. For epidemiological purposes an attempt was made to divide the material into biotypes. It soon became evident that some otherwise typical strains were capable of hydrolyzing urea, which raised the question of their proper taxonomic position.

The negative urease reaction is generally considered to be the main feature distinguishing *P inconstans* from the other *Proteus* species. However urease producing strains of *P inconstans* were recognized by *Stuart et al* (25) in one of the earliest studies of this bacterium, at that time named *Paracolon* Type 29911 *Ewing et al.* (8) and *Singer & Bar-Chay* (23) reported strains with weak urease reaction. *Buttiaux et al.* (3 4) stressed the close resemblance between *P inconstans* and *P rettgeri* and suggested that intermediate strains should be regarded as *P rettgeri* if they hydrolyzed urea. The same principle seems to have been adopted in the diagnostic tables of *Edwards & Ewing* (7) where the urease reaction constitutes a clear cut difference between the two species. However there has been no general agreement about the significance of the urease reaction. Several authors have mentioned urease positive strains of *P inconstans* e g *Shaw & Clarke* (22) *Sen* (21) *Carpenter* (5) and *Kholodkova* (12)

The present paper gives an account of the biochemical reactions of 108 strains from the collection mentioned above. They were selected according to principles specified in detail below In view of the unsettled taxonomic status of the urease positive strains, 18 typical strains of *P rettgeri* were included in the study for comparison.

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MATERIAL AND METHODS

Strains

Most of the strains were isolated from urine or chronic wounds from patients in long-term wards.

adsorbed to protein A-containing staphylococci. Streptococci not belonging to groups A, B, C, D and G were excluded. The group D strains used all produced black colonies on the McLeod medium containing potassium tellurite in a concentration of 1:2500, thus belonging to the *Streptococcus faecalis* group (18).

Agglutination of Sensitized Sheep Red Cells by Streptococci

The ability of streptococci and streptococcal preparations to agglutinate sensitized sheep red cells was determined by a procedure essentially similar to the methods described by *Sjöquist and Stålenheim* (17) and *Winblad and Ericson* (21) for the detection of protein A in staphylococci. In these methods, cell wall associated protein A is regularly detected by slide agglutination of staphylococci using 3 per cent sensitized sheep red cells, while soluble protein A is detected in tubes or Microtiter plates with 0.25–1 per cent cells (21). Since slide agglutination of sensitized sheep red cells by streptococci is often difficult to discover with the naked eye, mainly because of auto-agglutination of the streptococci, the Microtiter technique was adopted for examining streptococci.

The sediment from a 10 ml Todd Hewitt broth culture grown over night was washed once in phosphate buffered saline (PBS, 0.12 M NaCl, 0.03 M phosphate, pH 7.2) and suspended in 0.1 ml volume of PBS. 25 µl of such suspensions were serially diluted 1:2, 1:4 and so forth ending with 1:4096 in Microtiter plates in PBS containing 0.5 per cent human albumin. 25 µl sensitized sheep red cells suspended to a concentration of 0.25 per cent in PBS was then added to each well and the sedimentation pattern recorded at 18 h. In some cases haemolysis made it difficult or impossible to read the test results. Incubation of these particular A, C and G strains for 30 min at 56° C before testing prevented such haemolysis. This treatment did not affect the capacity of the streptococci to agglutinate sensitized sheep red cells (see below).

Enzymatic Treatment of Streptococci

Trypsin-treatment. Streptococci, belonging to groups A, B, C, D and G were grown over night in Todd Hewitt broth. The sediment from a 10 ml culture broth was washed in 0.4 M Tris (hydroxymethyl) aminomethane (Tris) pH 8.0 and suspended in 0.2 ml of Tris buffer. Trypsin solution, 0.06 ml (15 mg per ml, Sigma) was then added (giving a final trypsin concentration of 0.45 mg/ml) and the suspension was incubated at 37° C.

The capacity of streptococci to agglutinate sensitized sheep red cells with and without trypsin was determined before digestion was initiated. After incubation, over night at 37° C the bacteria with trypsin and without were washed 3 times in Tris, and tested again for the capacity to agglutinate sensitized sheep red cells.

Pepsin treatment of streptococci. Streptococci, belonging to groups A, B, C, D and G were grown over night in 10 ml Todd Hewitt broth. The sediment was suspended in 0.1 ml 0.5 N acetic acid, the pH was adjusted to 2.5 and 0.1 ml pepsin (Sigma), 10 mg/ml, was added. After incubation at 37° C for 4 h, 0.3 ml of 0.5 M Na_2HPO_4 was added for neutralization, followed by washings, 3 times, and suspension in 0.1 ml PBS. Controls with distilled water instead of pepsin were included.

Protease treatment of streptococci. Protease treatment followed the procedure for extraction of streptococcal group polysaccharide with pronase B (6). Protease from *Streptomyces griseus* (Sigma, protease type VI) was dissolved in borate buffer pH 7.5 with 0.01 M $CaCl_2$, to a concentration of 20 mg/ml. The sediment from an over night culture of streptococci in 5 ml Todd-Hewitt broth was suspended in 0.5 ml of the protease solution, and a control sediment of the culture suspended in 0.5 ml borate buffer. The Microtiter test was made before incubation, and after incubation at 37° C two h when washed 3 times in PBS and suspended in 0.1 ml PBS.

RESULTS

Agglutination of Sensitized Sheep Red Cells by Streptococci

In all, 268 isolates of streptococci belonging to Lancefield groups A, B, C, D and G were screened with the Microtiter technique for their ability to agglutinate sensitized sheep red cells. Agglutination of streptococci with sensitized sheep erythrocytes was seen within 2 h of sedimentation but the tests were finally read after 18 h. Spontaneously agglutinating streptococci were tested as easily as non spontaneously agglutinating strains. Haemolysis of the erythrocytes was not common and when it occurred it was easily prevented by a short incubation of the suspension at 56° C for 30 min.

The proportion of streptococci of Lancefield groups A, B, C, D and G which agglutinated sensitized sheep red cells is seen in Table 1. 97 per cent group A strains tested, 88 per cent of group C and 100 per cent of group G did agglutinate. There was a notable difference of the frequency of agglutinable strains in groups A, C and G on

TABLE 1 *Behaviour of Strains in Different Tests*

	P inconstans A 8 strains		*P inconstans B* 83 strains		Intermediate group 14 strains		*P rettgeri* 18 strains		Individual strains		
	+	(+)	+	(+)	+	(+)	+	(+)	70141	G 82	103
ility	8		61		13		17		+	—	+
rate	7		76		14		16		+	—	+
assium cyanide	8		83		14		18		+	+	+
rate reduction	8		83		14		18		+	+	+
ease	0		0		14		18		—	+	—
lole	8		74	4	14		18		+	+	+
nylalanine	8		83		14		18		+	+	+
latin (22° C)	0		0	1	0		0		—	—	—
cose (gas)	8		0		0		0		—	—	—
cose (acid)	8		83		14		18		+	+	+
cenose	0		0		0		6		+	+	—
ose	8		83		14		18		+	+	+
lactose	0		83		14		18		+	+	+
ctose	8		80	3	14		18		+	+	+
anose	8		81		14		18		+	+	+
bose	0		1		0		0		—	—	—
lobiose	0		0	4	0		0		(+)	—	—
rose	0	8	13	68	5	9	3	3	(+)	(+)	(+)
halose	0		83		14		0		—	—	+
nitol	8		1		0		18		+	—	+
lbitol	0	5	0		0		10	6	+	(+)	—
ythritol	0		0		0		15	3	+	—	+
cerol	0	7	74	9	12	2	10	8	(+)	—	+
sitol	0		83		14		17		+	—	+
nnitol	0		0	1	0		18		+	+	+
bitol	0		1	9	0	1	0		—	—	(+)
ortin	0		0		0		17		+	+	—
alicin	0		0	1	0		18		+	+	—
ocin	0		0		0		17	1	+	+	—

Positive reaction within 1–2 days' incubation +
Positive reaction within 3–7 days' incubation (+)
Negative reaction within 7 days' incubation —
All strains were negative in the following tests Hydrogen sulphide, malonate, Voges-Proskauer beta galactosidase, arginine, lysine, ornithine, arabinose, xylose, lactose, maltose, melibiose, melezitose, raffinose, dulcitol, amygdalin.

The ability to hydrolyse urea was reproducible and was retained during storage of some strains for more than two years.

Other Biochemical Tests

The biochemical reactions are shown in Table 1

There was agreement between the methods for the citrate test.

The behaviour of *P inconstans A* and *B* and *P rettgeri* was consistent with the current descriptions of the species (6, 7 11 20) The *P inconstans A* isolates were all galactose negative even after incubation over 14 days. They were also trehalose negative and erythritol negative.

Only few of the divergent biochemical reactions exhibited by some *P inconstans B* strains were present in one and the same strain. Among the indole negative isolates, one was also sucrose negative. Among the

TABLE 1 *The proportion of Streptococcal Isolates Belonging to Groups A B C D and G Agglutinating Sensitized Sh ep Red Cells.*

	Lancefield group of streptococcal strains tested					Total
	Group A	Group C	Group G	Group B	Group D	
Number of strains tested	123	16	15	57	57	268
Number f strains agglutinating sensitized sheep red cells	119/123	14/16	15/15	27/57	19/57	194/268

one hand, and in groups B and D on the other hand. The frequencies among the latter were 47 and 33 per cent, respectively Although this method did not allow to quanti tate the agglutinating capacity of the indi vidual isolate it appeared, however that group D streptococci in general contained very little activity in comparison to strains of groups A, B C and G (Compare also Table 2)

50 strains of streptococci belonging to groups A, B C, D and G were tested for agglutination of non-sensitized sheep red cells with the Microtiter technique In no case was any agglutination seen.

The capacity of streptococci to agglutinate sensitized sheep red cells was also studied in experiments in which rabbit serum was added. The free rabbit immunoglobulins will combine with the streptococci and thereby inhibit agglutination of the sensitized sheep red cells. A streptococcus group A strain was grown over night in 10 ml Todd Hewitt broth, washed in PBS suspended in 0.5 ml PBS and diluted as described above. 25 µl of serum from a non immunized rabbit diluted 1 10 1 100 or 1 1000 was then added to each streptococcal dilution followed by 25 µl of 0.25 per cent sensitized sheep red cells. Rabbit serum diluted 1 1000 produced no visible inhibition of the agg utination. Rabbit serum 1 100 reduced the agglutinating titer of the streptococci to one-half serum diluted 1 10 reduced the titre to one fourth. Inhibition by similar dilutions of group specific anti-A rabbit serum (Wellcome) caused the same inhibition

The Capacity of Streptococcal Extracts to Agglutinate Sensitized Sheep Red Cells

Extraction of groups A, B C D and G at pH 1.5 2.5 4.5 7.2 and 10 0 was performed in water bath at 100 C for 10 and 60 min. Extraction and neutralization procedures and the results are summarized in Table 2. Agglutination of sensitized sheep red cells by extracts was seen in dilutions ranging from 1.2 to 1.32. Lancefield extract at pH 1.5 of a group A strain, made as described in Table 2, agglutinated sensitized sheep red cells in a 1.2 dilution, while a similar extract of a group G strain agglutinated in a 1 4 dilution. The strongest effect was obtained with extracts made at pH 10.0

Stability of Streptococcal Factor Agglutinating Sensitized Sheep Red Cells

The following procedure had no effect on the agglutinating capacity subculturing, storage for 10 days at room temperature of extractions made at pH 10 and adjusted to pH 8 and incubation until loss of viability

Effect of Procedures which Inactivated Streptococcal Enzymes and Streptolysins on Sensitized Sheep Red Cell Agglutinating Factor

It is wellknown that various proteolytic enzymes agglutinate certain immunoglobulin

TABLE 2. *The Results of Biochemical Reactions of the Two β-glucuronidase N gative Group B Strains*

	Bovine strain	Human strain
Haemolysis on sheep blood-agar	–	+
Camp on sheep blood-agar	+	+
Bacitracin inhibition zone of growth	–	–
Salicin	+	+
Mannitol	–	–
Inulin	–	–
Lactose	+	–
Raffinose	–	–
Trehalose	+	+
Sorbitol	–	–
Sodium hippurate	+	+
Aesculin	–	–
Litmus milk		
Acid	+	–
Coagulation	+	–
Reduction	–	–

The former belonged to type X (9829 NCTC) the latter had been classified as Lancefield's type II but crossreacted with type R antiserum (9826 NCTC) (5)

The results of biochemical reactions of the two β-glucuronidase negative group B strains were in no respect irregular compared to the other group B strains studied by *R. Holth Haug* (3)

It was estimated from the standard curve that samples containing 1 phenolphthalein unit or more of β-glucuronidase could be detected colorimetrically Ninety-eight of the hundred group B samples contained at least 10 phenolphthalein units of β-glucuronidase, and ninety-four of these contained at least 40 phenolphthalein units.

DISCUSSION

The method of *Jacox* (5) was selected for the present study Working with 529 strains of group A, 16 per cent were found to be β-glucuronidase positive. All 36 strains of group A, type 28 showed positive reaction. *Johnson et al.* (12) and *Williams* (15) using other methods, found somewhat lower percentages of positive reaction among strains of group A (4 per cent and 11 per cent, respectively)

Jacox (5) found β-glucuronidase to be present in 23 of 42 strains from groups B C, G and L, among which 4 of 6 group B strains contained the enzyme. Lack of detectable β-glucuronidase production in only two of our 100 group B strains suggests that nearly all group B strains are capable of producing the enzyme. β-glucuronidase might furthermore be demonstrable in the last two group B strains with other growth conditions, as preliminary tests seem to indicate the two strains gave a moderate β-glucuronidase reaction when the bacteria were harvested from colonies grown on agar plates of Mueller Hinton Medium (Difco)

In a material of 50 unspecified strains named "enterococci" *Hawksworth et al* (4) found a mean value of 2.9 ± 0.6 μmoles phenophthalein glucuronidase degraded per hour per 10^9 cells. The value was considered to be low as compared to those found in other bacteria. The corresponding value is calculated to be maximum 0.0004 μmoles in the present study assuming the samples to contain at least 10^9 cells.

Group D strains did not show positive β-glucuronidase reaction when harvested from Mueller Hinton agar plates.

The present group D material includes *S faecalis S faecalis* var *zymogenes S faecalis* var *liquefaciens S faecium* and *S faecium* var *durans* which comprise the enterococci according to *Hartman et al.* (2) Better knowledge of β-glucuronidase occurrence in other group D streptococci, such as *S bovis* and *S equinus* might be of value to streptococcal classification.

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TABLE 2. *The Capacity of Extracts of Streptoc*

Extraction buffer	pH of the extraction buffer	Neutralisation buffer
0.15 ml 0.2 N HCl	1.5	0.45 ml 0.5 M Na_2HPO_4
0.15 ml 0.5 N Acetic acid	2.5	0.45 ml 0.5 M Na_2HPO
0.3 ml 0.1 M Acetic buffer	4.5	0.3 ml 0.5 M Na_2HPO_4
0.6 ml PBS	7.2	—
0.3 ml 0.2 N NaOH	10.0	0.3 ml 0.1 M Acetic buffer

Bacterial pellets from 10 ml Todd Hewitt broth cultures grown over night were heated at 100° C tested for agglutination of sensitized sheep red cells in Microtiter plates.

covered red cells, also sensitized sheep red cells. Streptococci are known to possess numerous enzyme activities. Most enzymes are thermolabile (13) and heating destroys also streptolysins (8 10)

Three group A isolates, three group B one group C and three group G isolates were grown over night on 10 ml Todd Hewitt broth washed in PBS and suspended in 0 1 ml PBS Heating of the suspension for 30 min at 60 C did not result in a decreased co-agglutination. Heating for 15 min at 100 C suppressed the activity of one group G strain but not of any of the other strains. Heating at 60 C for 30 min and at 100 C 15 min of 16 group D strains, increased the agglutinating capacity of 11 strains, resulting in just as strong agglutination as occurred among groups A,B C and G Five group D strains were unaffected by heating

Deoxyribonuclease and lipoprotease are relatively thermostable (11 20) Deoxyribonuclease is inhibited by binding Ca^{++} and Mg^{++} with EDTA 0.03 M (19) while lipoprotease is destroyed by trypsin (11) Two group A strains and one group G strain were suspended in 0 05 M EDTA. Their ability to agglutinate sensitized sheep red cells was, however unaffected. Nor did trypsin abolish the activity (see below)

Streptokinase tolerates exposure to 100 C for 30 min (13) but the fibrinolytic activity of streptokinase is inhibited by epsilon aminocaproic acid (EACA) at a concentration of 1 mg/ml (4) 2 group A strains and one group G strain were suspended in EACA (1 mg/ml) and incubated at 37 C for one h without any consequent change in reactivity with sensitized sheep red cells

Effect of Streptococcal Protease on the Agglutination of Sensitized Sheep Red Cells

To investigate the influence of streptococcal protease on the agglutination of sensitized sheep red cells the following experiment was performed. Group A streptococci were grown over night on Todd Hewitt broth (10 ml) The culture was afterwards centrifuged and the sediment was reincubated in 2 ml Todd Hewitt broth together with 0 4 ml 50 per cent glucose After 16 h the pH in the culture was 5 8, sodium thioglycolate was added in a final concentration of 0 1 M and the culture was incubated for 1 h at 37 C.

The ability to clot milk (7) after 15 min incubation with milk and sodium thioglycolate in a final concentration of 0 1 M was then established. The agglutination of sensitized sheep red cells was found not to be influenced by the presence of protease in the bacterial medium when compared with a similar concentration of bacteria alone in PBS

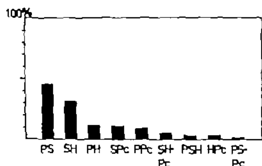


Fig 3 Period prevalence rate for the simultaneous isolation of 2 or more of the 4 predominating bacteria per patient P *Pseudomonas aeruginosa*, S *Staphylococcus aureus* H *Haemophilus influenzae* Pc *Diplococcus pneumoniae*

from pts. harbouring *St aureus* chronically (14 per cent) ($p<0.05$) whereas no significant differences were found as regards the other species.

Fig 4 shows the *period prevalence rates* of the 4 predominating species, Enterobacteriaceae, and other members of the miscellaneous group for different age groups of pts. It is seen that there is no major difference between the different age groups. From early life, CF pts. are subject to colonization, even chronically with the usual species and any of these can be isolated as the initial, colonizing bacteria. Although the *period prevalence rate* of *Ps aeruginosa* seems to be increasing

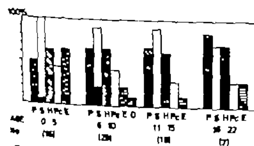


Fig 4 Period prevalence rate of the 4 predominating bacteria (P S H, Pc, see text to Fig. 3) Enterobacteriaceae (E) and other members of the miscellaneous group (O) (see Table 1) in the respiratory tracts of 70 cystic fibrosis patients classified into different age groups (years) The black part of the columns represents chronic colonization. Figures in parentheses indicate number (No.) of patients in each age group.

with age in contrast to the slight decrease of *St aureus* as well as *D pneumoniae* these differences are insignificant, whereas the decrease of Enterobacteriaceae is significant ($p<0.01$)

No significant difference between the 2 sexes was found as regards the occurrence of any of the species.

DISCUSSION

In accordance with other reports (1 4 6 12, 18, 29 30) the results show that the predominating organisms in the respiratory tracts of CF pts. are the 4 species *St aureus Ps aeruginosa H influenzae* and *D pneumoniae* but other bacteria, especially Enterobacteriaceae also colonize these pts. These results underline that the problems of prevention and treatment of bacterial infection in the respiratory tract of CF pts. are not satisfactorily solved as these pts. are colonized recurrently and chronically some of them from the very beginning of life. In spite of extensive treatment, the pattern of the respiratory tract bacteriology remains characteristic throughout life in these pts. (Fig. 4) The complicated fluctuations of the bacteriology which are described by means of epidemiological terms in this study have not been reported in detail before (Fig. 1)

The "daily impression" from the work in the bacteriological laboratory—and the results which are comparable with other reports on the use of "isolation rate"—is probably expressed in the *point prevalence rates* (Fig 1 and 2) from which it appears that *Ps aeruginosa* and *St aureus* are the predominating organisms.

From an epidemiological point of view however a more true picture is obtained by a combination of the data from the other "*rates*" (Table 1 and Fig. 1) The *period prevalence rates* show that during one year the main part of the pts. at some time harbour *St aureus Ps. aeruginosa* is not quite as frequent but equals *H influenzae D pneumoniae* and the miscellaneous group are, however by no means negligible, especially in

...ains t Agglutinate Sensitized Sheep Red Cells.

...traction ...e in ...n	The highest dilution giving agglutination				
	2 group A strains	2 group B strains	1 group C strain	2 group D strains	1 group G strain
10		0	0	0	4
60	2	0	0	0	2
10	4	2	2	0	8
60	0	2	0	0	16
10	4	0	0		4
60	2	2	0	0	8
10	0	0	2	0	4
60	8	0	0	0	2
10	0	0	0	0	0
60	32	8	32	2	32

...) or 60 minutes in extraction buffers, then neutralized and centrifuged. 25 µl of each supernatant was

Treatment of Streptococci with Proteolytic Enzymes

Two isolates of each groups A, B C, D and G were digested with proteolytic enzymes. Neither trypsin nor pepsin treatment had any influence on the factor which agglutinated sheep red cells in any strain tested

Treatment of streptococci with protease from *Streptomyces griseus* however resulted in a marked decrease of activity after 2 h incubation of one isolate of each of the groups A, B, C and G while one group D strain remained unaffected

DISCUSSION

In this investigation the agglutination of sensitized sheep red cells by streptococci was confirmed and the capacity was demonstrated not only in streptococci of groups A C and G (12) but also in group B and D streptococci. The Microtiter technique using dilutions of streptococci can not be used to quantitate the sensitized sheep red cell-agglutinating capacity on the bacterial surface because of steric inhibition each streptococcal cell can only bind a limited amount of sensitized sheep red cells. For example, the unaltered agglutination after treatment of streptococci with trypsin and pepsin does not exclude that the factor may be somewhat susceptible to this treatment. It only shows that the capacity is not abolished. On the other hand, the strongly diminished agglutinating activity after treatment with protease from *Streptomyces griseus* indicates high susceptibility to this enzyme. The factor responsible for the agglutination may perhaps, be of protein nature as it is sensitive to protease from *Streptomyces griseus* (16) Since the capacity to agglutinate sensitized sheep red cells is not abolished by trypsin pepsin or heating it has obviously no connection with M protein or T-antigen (15) The unaltered agglutination after heat treatment rules out any connection with the active sites of heat labile enzymes and streptolysins. The following enzymes are destroyed or their ability is decreased on exposure to 60 C for 30 min, a procedure, which will abolish even streptolysin activity (8 10) ATP-ase (9) N-acetylglucosaminidase (9) amylase (3) dehydrogenases (14) esterase (9) alpha-glucosidase (5) hyaluronidase (13) NAD-glycohydrolase (1) phosphatase (9) and protease (13) Blocking procedures for heat stable streptococcal enzymes did not have any effect either The capacity of streptococci to agglutinate sensitized sheep red cells but not non-sensitized sheep red cells indicates a binding between the streptococci and the rabbit immunoglobulin, which has its antibody combining sites attached to the sheep red cell antigens. This capacity can be characterized as "non-

ede the respiratory tract with the 4 predominating species (4 9 17 18) Recently *Høiby & Axelsen* (1973) have proposed that the immune response of these pts. could be responsible for the selection of the mucoid strains of *Ps aeruginosa* characteristic for these pts. i.e the mucoid substance should favour the mucoid strains at the expence of the non-mucoid strains by inhibiting the opsonizing effect of antibodies on the bacterial cells and by inhibiting the complement dependent lysis of the bacterial cells (13) Moreover a type III hypersensitivity reaction between the antibodies and the bacterial antigens was proposed as a factor contributing to the tissue damage (14, 15)

Similar *mechanisms could possibly be* working with respect to *St aureus*. As shown by *Jensen* (1959) most strains of *St. aureus* have protein A in the cell wall. Protein A has been shown to bind immunoglobulins in the Fc part (32) and this effect has been shown to inhibit phagocytosis by polymorphnuclear leucocytes (5) Moreover complexes of protein A and human immunoglobulins can activate the complement system, but this does not lyse the *St. aureus* cells (33) These complexes are able to initiate localized Arthus-like reactions in laboratory animals and to liberate vaso-active amines (8, 26)

The reason why *St. aureus* and mucoid strains of *Ps aeruginosa* are the most prominent pathogens in CF pts. should according to these speculations be that these bacteria, by means of protein A and the mucoid substance, are more resistant than other species to the defence reactions in the respiratory tracts of these pts. Moreover Arthus-like inflammatory reactions between components of these bacteria and the immunoglobulins might contribute to the tissue damage and possibly also explain why these bacteria seldom, if ever spread outside the respiratory tract in CF pts.

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immune" like the staphylococcal protein A–Fc reaction, but care should be taken not to confuse these two properties since they obviously differ in chemical behaviour the protein A being very susceptible to trypsin. In addition the binding sites of immunoglobulin G seem to be different (12)

The possible participation of the factor responsible for the agglutination of sensitized sheep red cells in serological grouping and typing of streptococci should be considered. Dependent of the treatment of the streptococci used for absorption of undesired activity of typing and grouping sera the specific titer can diminish if the factor is present under the absorption procedure. The presence of the factor agglutinating sensitized sheep red cells, in extracts of streptococci may cause non-specific reactions in precipitation test.

The biological significance of the factor is so far unknown but is subjected to studies.

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TABLE 1 *Occurrence of* Pseudomonas aeruginosa *in the Respiratory Tract and the Occurrence of* Pseudomonas aeruginosa *Precipitins in Sera from 70 Cystic Fibrosis Patients*

	No. of pts.	No. and percentage of pts. with *Ps. aeruginosa* precipitins	Mean ($\bar{x}$) and range (r) of no. of precipitins per pt. with *Ps. aeruginosa* precipitins	
CF + P (Total)	45 (64%)	29 (64% c.l.: 78%–49%)	$\bar{x}$ 14	1–50
CF — P	25 (36%)	2 (8% c.l.: 26%–1%)	$\bar{x}$ 1	r 1
CF + NMP(i)	13 (29%)	1 (8% c.l.: 36%–0%)	$\bar{x}$ 1	
CF + NMP/MP(i)	5 (11%)	2 (40% c.l.: 85%–5%)	$\bar{x}$ 1	r 1
CF + MP(i)	0			
CF + NMP(c)	1 (2%)	1	$\bar{x}$ 8	
CF + NMP/MP(c)	5 (11%)	5 (100% c.l. 100%–48%)	$\bar{x}$ 6	3–12
CF + MP(c)	21 (47%)	20 (95% c.l. 100%–76%)	$\bar{x}$ 18	r 4–50

One-year period prevalence and period prevalence rate of the occurrence of *P. aeruginosa* in the Respiratory tracts of 70 cystic fibrosis patients. Also the occurrence and number (no.) of *Ps. aeruginosa* precipitins in sera from the same patients are given. The patients have been classified into groups of subjects according to the bacteriological findings. For explanation of abbreviations used see Materials and Methods. 95 *per cent confidence limits is abbreviated* c.l.

Statistical Methods (5, 21)

Quantitative data. The Mann-Whitney test. Qualitative data: χ^2-test with Yates correction if indicated or non-parametric rank sum test for comparison of series of frequencies. *Correlation calculations.* Spearmans correlation coefficient R.

RESULTS

The results of the monthly bacteriological examinations as regards *Ps. aeruginosa* during the one-year period are given in Table 1. Nearly 2/3 of the pts. have harboured *Ps. aeruginosa* and, among these, 69 per cent harboured mucoid strains (95 per cent confidence limits 53 per cent—82 per cent) whereas 31 per cent harboured solely non-mucoid strains.

There was no differences between the two sexes as regards the period prevalence rate of *Ps. aeruginosa* or the period prevalence rate of mucoid strains or chronic colonization.

In Table 2 the 45 pts. who harboured *Ps. aeruginosa* have been classified into 2 groups: one group comprising pts. who chronically harboured *Ps. aeruginosa* and another group comprising pts. who intermittently harboured *Ps. aeruginosa*. It is seen that in the group who chronically harboured *Ps. aeruginosa* mucoid strains were predominating, whereas non-mucoid strains were predominating in the group of patients who only intermittently harboured *Ps. aeruginosa*. This difference is significant ($p < 0.0001$).

The occurrence of precipitins against *Ps. aeruginosa* in sera from the 70 pts. classified into different groups of subjects according to the results of the bacteriological examinations are given in Table 1. Examples of

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CANINE MYCOPLASMAS II BIOCHEMICAL CHARACTERIZATION AND SEROLOGICAL IDENTIFICATION

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A total of 118 mycoplasma strains isolated from the conjunctivae and the respiratory and genital tracts of 78 dogs were studied biochemically and serologically Three biochemical tests (glucose fermentation, catabolism of arginine and phosphatase activity) were found t be very useful for a preliminary biochemical grouping prior to serological identification. Using these tests, the strains could be divided into six groups that did not show any anti genic overlapping. By serological examination using the indirect immunofluorescence technique and growth inhibition 35 isolates were identified as *M canis* 21 were identified as *M edwardii*, 10 as *M cynos* 10 as the f line species *M gateae* 30 as *M spumans* 2 as *M maculosum* 2 as the bovine species *M bovigenitalium* and 5 as *M felimin* turn a species previously represented by a single strain isolated from a cat. The three remaining strains which were glucose positive and arginine and phosphatase negative were placed in a separate serogroup group A, as they were serologically different from the established canine species.

The isolation of mycoplasmas from the conjunctivae and the respiratory- and genital tracts of dogs was described in a previous publication (13) A total of 118 mycoplasma strains were isolated from 78 dogs. The pur pose of the present study was to characterize and differentiate the strains according to biochemical properties and subsequently to identify the strains serologically

MATERIALS AND METHODS

A. Biochemical Studies

I. *Myc plasmas.* In addition to the 118 wild strains, the type strains of the following canine *Mycoplasma* species were examined *M spumans* (PG 13) *M canis* (PG 14) *M maculosum* (PG 15) *M edwardii* (PG 24) and *M cynos* (H 831) All strains were cultivated on B-medium (13) Initially the strains were cloned by picking single colony from a plate inoculated with the highest culture dilution producing growth. The colony was crushed and suspended in fluid medium from which ten-fold serial dilutions were streaked onto plates. This procedure was repeated three times.

II. *Tests Fermentation of glucose and mannos* A base medium of the following composition was used: Heart infusion broth (Difco) 2.5 per cent (w/v) 90 ml; PPLO serum fraction (Difco) 1 ml sterile distilled water 29 ml thallium acetate, 10 per cent (w/v) 1 ml Na penicillin, 50,000 i.u. DNA (Sigma) 0.2 per cent (w/v) 1. ml and phenol red 0.06 per cent (w/v) 5 ml. In order to remove traces of glucose and arginine, the heart infusion broth was treated with glucose oxidase peroxidase, and arginase (Sigma) (14) pH was adjusted to 7.8. The test substrates were made by adding 1.6 ml of 50 pe cent (w/v)

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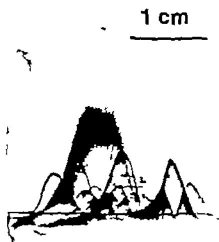


Fig 2 Crossed immunoelectrophoresis of 2 µl St-Ag against patient's serum (15 µl/cm²) in the second dimension gel. 50 precipitates could be seen in this plate. The patient was chronically colonized with mucoid *P aeruginosa*. (Electrophoresis and staining as in Fig. 1)

and mucoid strains and most of the pts. intermittently colonized with *Ps aeruginosa* in the respiratory tract had no precipitins.

Fig. 3 illustrates the relationship between the number of *Ps. aeruginosa* precipitins per serum and the duration of the chronic *Ps. aeruginosa* colonization in those 24 pts. where the duration of the period of chronic colonization was known. This correlation is weak though positive and significant as regard males but not as regard females (p and R values are given in the figure)

There was a significant difference between the two sexes in the group of pts. chronically colonized with mucoid strains of *Ps aeruginosa* as regards the number of precipitins per serum ($p < 0.02$) the 12 males having, on an average, twice as many precipitins per serum (mean 22, range 0–50) as the 9 females (mean 11 range 4–16) No difference in the age of the pts. could probably account for this phenomenon (males, mean 11.6 years, range 1.5–22 years, females, mean 9.6 years, range 2.5–17.5 years) According to Fig 1 the difference in duration of the chronic *Ps. aeruginosa* colonization could possibly not account for this difference in the number of precipitins either (males, mean duration 2.6 years, range 0.5–4 years, females, mean duration 2.2 years, range 0.5–4 years)

DISCUSSION

The period prevalence rate of *Ps aeruginosa* and the predominance of mucoid strains observed in this study is in accordance with other reports on CF bacteriology (4 6–9 11 16 17) and contrast with most other groups of pts. not having cystic fibrosis in whom mucoid strains only account for 1 per cent–3 per cent (7–9 18) However in bronchiec

TABLE 3. *O currence of Mucoid and Non-mucoid Strains of* Pseudomonas aeruginosa *in the Respiratory T act and the S bseq ent* Occurrence of Pseudomonas aeruginosa *Precipitins in Sera f om 10 Cystic Fibr sis Patients Harbouri g* Pseudomonas aeruginosa *in the R spi atory T act for the First Time*

No. of patients	MP or NMP first isolated	Subject group at the end of the study	No. of precipitins
6	NMP	CF + NMP(l)	0
1	NMP	CF + NMP/MP(l)	1
1	NMP	CF + MP(c)	16
1	MP	CF + NMP/MP(c)	0
1	MP	CF + MP(c)	14

Results of the first isolation of *Ps. aeruginosa* as regards mucoid or non-mucoid strains in 10 cystic fibrosis patients who previously never had harboured *Ps. aeruginosa* in the respiratory tract. Besides, the results of the subsequent bacteriological examinations are summed up together with the subsequent occurrence of *P aeruginosa* precipitins. For explanation of abbreviations used see Materials and Methods.

stock solutions of glucose and mannose. The test substrates were inoculated with a single colony. The reaction was read by comparison with inoculated and uninoculated base medium as well as with uninoculated test substrate. Positive reaction was recorded when a distinct colour difference (red-yellow) between the inoculated test substrate and each of the control substrates was present; a negative reaction was recorded when no distinct colour difference was seen. The reactions were followed during incubation at 37 C for 14 days. Subcultivation with another set of test substrates and controls was performed on days 4 and 8, and at the same time growth was checked by streaking 0.01 ml onto B-plates. Glucose fermentation was examined both under aerobic and anaerobic conditions, whereas mannose fermentation was examined aerobically only. Anaerobic conditions were provided by adding an overlay of 1 ml of melted sterile vaseline and paraffin mixture (equal parts).

Catabolism of arginine and urea. The same base medium as for fermentation of glucose and mannose was used, except that pH was adjusted to 7.3. The test substrates consisted of base medium with the addition of 4.25 ml L-arginine (Sigma) and 5.8 ml urea from 50 per cent (w/v) stock solutions. Test substrates and base media were inoculated with single colonies. Test procedure and reading of reactions were as described for glucose fermentation.

Phosphatase activity. Plates containing phenol phthalein disphosphate (Sigma) were prepared according to *Aluotto et al.* (1). Three plates were inoculated with an inverted agar block containing growth. The agar block was removed and the plates incubated at 37 C for 3, 7 and 14 days, respectively. 5N NaOH was flooded on the agar surface and the immediate appearance of a red colour indicated a positive reaction.

Formation of film and spots. This phenomenon was examined on medium containing egg yolk (5). The plates were inoculated by the inverted block method and incubated for 1 week at 37 C followed by 1 week at room temperature.

Serum digestion. The ability of the strains to digest serum was tested on a medium containing 75 per cent coagulated horse serum (1). The surface of the medium was inoculated with cultures containing at least 10 colony forming units per ml (c.f.u./ml). During 14 days of incubation at 37 C the media were examined for liquefaction. For comparison, media were inoculated with *M. mycoides* subsp. *capri* (PG 3) known to be positive (6) to this test.

Tetrazolium reduction. This was examined in fluid B-medium at two different concentrations (0.009 per cent, w/v and 0.045 per cent, w/v) of 2, 3, 5-triphenyltetrazolium chloride. The substrates were inoculated with single colonies. Reduction was examined both aerobically and anaerobically for both concentrations. A positive reaction was recorded when pink or red colour developed during incubation at 37 C for 14 days. For comparison uninoculated controls were incubated simultaneously. Subcultivation and check for viability were done at days 4 and 8.

Haemolysis. The overlay technique was used (2). A five per cent blood agar (guinea pig erythrocytes) was added to a plate with 4 days old colonies. After another day of incubation at 37 C the plates were read. A greenish zone surrounding the colonies was recorded as α-haemolysis, a clear zone as β-haemolysis.

Haemagglutination. The cultures were examined undiluted and in serial twofold dilutions to 1:16 in PBS pH 7.4. The dilutions were prepared in a perspex agglutination tray. To 0.5 ml of culture was added 0.5 ml of a 0.5 per cent suspension of guinea pig erythrocytes washed three times. The reaction was left at room temperature and read when the blood cells were sedimented in the control wells with uninoculated medium.

Haemadsorption. Adsorption of guinea pig erythrocytes to mycoplasma colonies was tested by flooding agar plates containing 4 days old colonies with 2 ml of a 0.5 per cent suspension of erythrocytes in PBS pH 7.4. The plates were incubated for 30 minutes at room temperature upon which non-adsorbed blood cells were washed away with PBS. The haemadsorption reaction was read with a stereo microscope at 100 times magnification.

B. Serological Identification

I. *Antisera.* Antisera against the type strains of the following species were used: *M. spumans* (PG 13), *M. canis* (PG 14), *M. maculosum* (PG 15), *M. edwardii* (PG 24), *M. cynos* (H 831), *M. gateae* (CS), *M. bovigenitalium* (PG 11) and *M. feliminutum* (BEN). These sera were made by the FAO/WHO International Reference Centre for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus.

Antisera against strains H 11, H 201, H 372, H 542, H 764, and H 756 were produced in the following manner: Antigens for immunization were obtained by centrifugation of cultures grown in rabbit meat infusion broth (10). The sediment was washed and resuspended in PBS pH 7.4. The suspension contained approximately 10 to 10^{8} c.f.u./ml. At intervals of one day albino rabbits were given two subcutaneous and three intramuscular injections. Each injection consisted of 1 ml antigen + 1 ml alhydrogel adjuvans (Superfos Export Company A/S, Copenhagen). After an interval of six weeks, the rabbits were given 1.5 ml antigen intravenously and finally bled by cardiac puncture ten days later.

II. *Tests.* The strains were identified by the indirect immunofluorescence test (10). For each

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HAPTEN-CARRIER RELATIONSHIPS IN IMMUNOLOGICAL UNRESPONSIVENESS

II Decrease of Antibody Affinity and Specificity in B Cell Tolerance

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Mice were rendered tolerant to a haptenic determinant NIP by cyclophosphamide treatment and subsequent multiple intraperitoneal injections of NIP coupled to mouse serum albumin. Control mice received no antigen. The mice were challenged with immunogenic NIP conjugates 20 days after stopping the tolerance inducing treatment. The response to the hapten was reduced, while the response to the carrier was normal. A second challenge showed absence of memory cell development towards the hapten. To study antibody affinity in tolerance rats were rendered tolerant to NNP human serum albumin and challenged later with the tolerogen. The rats developed very little anti-NNP antibodies until 3 months after the tolerance inducing treatment. Then the affinity of antibodies was low in the tolerant group. A partial tolerance lasted one year which is remarkably long when compared to other works on the length of B cell tolerance. Tolerant animals had only weakly specific antibody to the tolerogen in contrast to the controls. M turation of antibody affinity was paralleled by increase in specificity in the control rats.

The immunological tolerance in the mouse is separable to tolerance of thymus derived T cells and of bone marrow derived B cells (8) The low zone tolerance described by *Mitchison* (28) has been demonstrated to be a T cell tolerance (29) The high zone toler ance involves also B cells (29) but affects generally only high affinity antibody producing cells (9 16, 24 34 37) Low affinity antibodies are often produced by tolerant animals (2, 9 18, 26) Hapten-protein con jugates can cause tolerance to the haptenic determinant (3 10 12, 15 21 31 36) *Golan & Borel* (15) found, that homologous mouse serum proteins coupled with DNP are capable of making mice tolerant to the hapten. In their experiment DNP-coupled mouse gamma globulin seemed to be responsible for the induction of the tolerant state. In the present study tolerance induction was attempted by using albumin coupled haptens. The study shows, that tolerance can be induced also by repeated injections of hapten-albumin conjugates to mice and rats. Tolerance resulted, whether the hapten was coupled to homologous or heterologous albumin. The rats were partially tolerant despite one year elapsing since the tolerance induction was stopped. This means, that B cell tolerance is not wholly reversible in the rat.

MATERIALS AND METHODS

NMRI mice were obtained from Bomholtgård, Denmark. Rats were Spraque Dawley strain grown in this laboratory. Mouse serum albumin was prepared from mouse serum by DEAE-cellulose fractionation (Schleicher & Schuell Selectacel 70 lot 1717) at 0.01 molar phosphate buffer pH 7.0

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biochemical group, the isolates were tested first with antiserum against canine species exhibiting identical biochemical properties. Strains not identified as canine species were subsequently tested with antiserum against other species sharing biochemical properties with the strains. If strains of a given biochemical group gave a weak or no reaction when tested with reference serum, they were tested with antiserum against a selected representative for these strains. In all cases, antiserum was used in 1 10 dilution and in the optimal dilution (i the highest dilution producing strong fluorescence in homologous titration)

Growth inhibition (3) studies were carried out in order to confirm the results of the immunofluorescence examination. For a number of strains it was only possible to obtain inhibition if the test was performed under suboptimal growth conditions. This applies especially to the *M canis* strains. Suboptimal growth conditions were provided by removing the yeast extract and reducing the content of horse serum from 20 to 2.5 per cent in the B medium.

The metabolic inhibition test was used to examine more thoroughly the relationship between *M gateae* strains. The test was performed as arginine hydrolysis inhibition (9)

Also the double immunodiffusion test was used to examine the antigenic relationship between *M gateae* strains. Antigen for this test was prepared from fluid culture. The culture was harvested by centrifugation. The sediment was washed twice in PBS pH 7 4 and resuspended in sterile distilled water. The suspension was placed in an ice-bath and sonicated for five one-minute periods (intervals covering one minute) by use of Branson Sonic Power equipment. The double immunodiffusion was performed in 1 per cent agar (Noble) in veronal-acetate buffer pH 7 3. The agar was poured on slides at a thickness of 2 mm. By use of a metal matrix, seven wells (one central and six surrounding) were cut in the agar. The distance between all wells was 8 mm. The central well was filled with antigen and the surrounding wells with serum. The test was kept t room temperature in a humid atmosphere and read every second day during ten-day period

RESULTS

A Biochemical Studies

All strains were found to be negative for serum digestion and catabolism of urea.

On the basis of glucose fermentation, catabolism of arginine and phosphatase activity the strains could be divided into six groups 1) Fifty nine strains were glucose positive arginine and phosphatase negative 2) ten were glucose positive, arginine negative and phosphatase positive 3) ten were glucose negative, arginine positive and phosphatase negative 4) thirty two were glucose negative, arginine and phosphatase positive 5) two were glucose negative, arginine negative and phosphatase positive and 6) five strains were glucose, arginine and phosphatase negative (Table 2) The results of the biochemical studies correlated with the subsequent serological identification are shown in Table 1

B Serological Identification

The results of the serological studies are summarized in Table 2.

1 Identification of glucose positive arginine and phosphatase negative strains By means of the immunofluorescence test, 35 isolates were found to give strong fluorescence with *M canis* (PG 14) antiserum

In growth inhibition studies, a potent antiserum against *M canis* (PG 14) was not able (except in nine cases) to inhibit the wild strains. Using antiserum against one of the isolates (strain H 11) 27 out of the 35 *M canis* strains together with the type strain (PG 14) were inhibited.

Using antiserum against *M edwardii* (PG 24) 21 isolates gave strong fluorescence in the immunofluorescence test. The same isolates were also inhibited in growth inhibition by this antiserum.

By means of antiserum against a representative strain (H 54?) for the three remaining strains of the glucose positive arginine- and phosphatase negative group it was found both in the immunofluorescence and the growth inhibition test that these strains formed a single serogroup Furthermore, in immunofluorescence tests strain H 542 did not cross react with any of the known canine type strains. This serogroup is therefore regarded as a separate group and temporarily named serogroup A

Natrium acetate $3H_2O$ 3 886 g Dinemal natrium 5.886 g Natrium chloride 6.80 g Calcium chloride $2H_2O$ 1 47 g and distilled water ad 1000 ml

TABLE 1 *Purification of CI and CIs and C1q from Euglobulin Prepared by Acid (A) and Neutral (B) Precipitation from Each 200 ml of the Same Pool of Human Serum*

Protein-fraction	Protein mg	Tot. No. of CI units	CIs units/mg protein	Purification (×) CIs	Purification (×) C1q	Recovery in per cent CIs	Recovery in per cent C1q
A. Acid EU	315	7673	24.4	1	1	100	100
A1. Sed.* fr. 1st ppt.† at 0.115 μ, 4° C	56.7	5033	88.8	3.6	3.0	65.6	53.5
A2. Sed. fr. 2nd ppt. at 0.115 μ, 4° C	38.7	4807	124	5.1	4.2	62.6	51.0
A3. Sed. fr. 3rd ppt. at 0.115 μ, 22° C	21.8	4500	206	8.4	7.4	58.6	51.0
A4. Supern. EDTA§ and ppt. at 0.02 μ, 4° C	6.1	3112	510	21		40.6	
A4. Sed. EDTA‡ and ppt. at 0.02 μ, 4° C	11.9	740	135		11.5		43.5
B. Neutral EU	10?	4658	45.7	1	1	100	100
B1. Sed. fr. 1st ppt. at 0.115 μ, 4° C	36.8	3744	102	2.2	~1	80.4	74.6
B2. Sed. fr. 2nd ppt. at 0.115 μ, 4° C	31.2	3954	127	2.8	2.4	84.9	73.2
B3. Sed. fr. 3rd ppt. at 0.115 μ, 22° C	22.2	3906	176	3.9	3.4	83.9	74.0
B4. Supern. EDTA and ppt. at 0.02 μ, 4° C	4	3113	778	17		66.8	
B4. Sed. EDTA and ppt. at 0.02 μ, 4° C	10.8	682	135		8.4		67.5

* Sed. = sediment.
† Ppt. = precipitation.
§ Supern. EDTA = supernatant after EDTA treatment.
‡ Sed. EDTA = sediment after EDTA treatment.

euglobulin solution under continuous stirring at 0° C. After dilution of 1:3 (ionic strength = 0.115 μ) the euglobulin was gently stirred for an additional 60 min period at 4° C. The fine precipitate formed was centrifuged down at 15000 *g* for 30 min at 4° C. In some experiments a centrifugal force of only 1000 *g* was used. This gave slightly decreased recovery but had the advantage that precipitates were easily suspended and more rapidly dissolved. Precipitates were solubilized in 0.5 M NaCl containing 0.005 M phosphate buffer pH 7.5 and 0.15 mM $CaCl_2$. Solubilization was usually continued for at least two hours under stirring.

Step 2. The solution was reprecipitated at an ionic strength of 0.115 μ and solubilized as in Step 1.

Step 3. All procedures of Step 2 were carried out at a temperature of 22° C and with centrifugation usually at 1000 *g*.

Step 4. The solution was diluted 1:2 with 0.02 M EDTA and left overnight at 4° C. After a further twofold dilution with 0.02 M EDTA the ionic strength of the solution was finally adjusted to 0.02 μ with 0.005 M phosphate buffer pH 7.5 (without $CaCl_2$) and left at 4° C for 60 min. The sediment after centrifugation at 15000 *g* for 30 min was dissolved as in Steps 1 to 3.

The stepwise purification of CI was analyzed on EDTA-treated precipitates by determination of CIs and C1q. Results of a typical experiment are given in Table 1. The subunits were purified closely in parallel and recovered at similar ratios indicating that CI, as could be expected, was maintained during Steps 1 to 3 as intact complex.

CI at about the same degree of purity was obtained from neutral EU and from acid EU despite the fact that the latter material was less pure.

At Step 3 CI was found to be freed of most proteins soluble at an ionic strength of 0.115 μ. The complex was dissociated at this stage by EDTA-treatment followed by a lowering of the ionic strength to 0.02 μ in order to precipitate C1q as well as contaminating serum components. The major part of CIs was recovered in the supernatant purified about 20-fold with respect to the esterase activity of euglobulin. The supernatant did not

TABLE 1 *Biochemical Properti*

Number of strains po

Species/Serogroup	Number of strains examined	Fermentation Glucose aerobic	Glucose anaerobic	Mannose	Catabolism of arginine	Phosph
M canis	36	36	36	34	0	0
M edwardii *	22	22	22	17	0	0
Serogroup A	3	3	3	3	0	0
M cynos *	11	11	11	11	0	11
M gateae	10	0	0	0	10	0
M spumans	31	0	0	0	31	31
M maculosum *	3	0	0	0	3	3
M bovigenitalium	2	0	0	0	0	2
M feliminutum	5	0	0	0	0	0

* The type strains are included.

2 *Identification of glucose positive arginine negative and phosphatase positive strains* The ten isolates in this group gave strong fluorescence with *M cynos* (H 831) antiserum Unfortunately the type antiserum did not contain growth inhibiting antibodies and thus, inhibition of the isolates of this species was not attempted.

3 *Identification of glucose negative arginine positive and phosphatase negative strains* This group consisted of ten strains using the immunofluorescence test, the strains were divided in two homogenous groups. Group 1 contained eight strains, strain H 372 being representative and group 2 contained two strains, strain H 201 being representative Strain H 372 and strain H 201 cross reacted both with the type strain of the feline species *M gateae* (CS) but not with each other (Table 3) Strain H 372 was also tested against other arginine positive type strains of human or animal origin, but no cross reactions were found.

In growth inhibition metabolic inhibition and double immunodiffusion strain H 372 strain H 201 and *M gateae* (CS) were examined for antigenic relationship The results appear from Table 3

4 *Identification of glucose negative arginine and phosphatase positive strains* In this group comprising 32 isolates, 14 strains (group I) were found to give strong fluorescence with *M spumans* (PG 13) antiserum 16 strains (group II) gave only weak or no fluorescence with PG 13 antiserum but strong fluorescence with antiserum against a representative strain (H 764) for this group. In growth inhibition tests all 30 strains were examined with *M spumans* (PG 13) antiserum as well as with antiserum against strain H 764 All the strains were inhibited by both sera, except that *M spumans* (PG 13) was inhibited only by homologous serum.

The remaining two strains of this biochemical group were identified as *M maculosum* both by immunofluorescence and by growth inhibition.

5 *Identification of glucose negative arginine negative and phosphatase positive strains* The two strains of this group gave strong fluorescence with antiserum against *M bovigenitalium* (PG 11) This antiserum also inhibited the strains in the growth inhibition test.

6 *Identification of glucose arginine and phosphatase negative strains* Using antiserum against a representative strain (H 756) and the indirect immunofluorescence test

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EXTRACTION OF CELL-BOUND HYALURONIDASE AND AMINOPEPTIDASE FROM *STREPTOCOCCUS MITIS*, ATCC 903

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Different methods for disintegration of *Streptococcus mitis* strain ATCC 903 were tested for extraction of hyaluronidase and aminopeptidase. Two methods were found effective, autolysis and freeze-pressing. Reproducible conditions for the extraction of both enzymes were autolysis for 20 hours at 37 C in 0.05 *M phosphate* buffer at pH 6.8 in the presence of chloramphenicol (100 μg/ml). The amounts of enzymes released *per unit of autolysed* cells were not affected by the incubation temperature. Disintegration by freeze-pressing reduced the specific activity of hyaluronidase by 70 per cent as compared to the autolytic procedure. Although the yield of aminopeptidase in extracts prepared by freeze-pressing was approximately equivalent to that in autolytic extracts, the stability of the enzyme was reduced.

previous paper on the occurrence of ...uronidase and aminopeptidase in cultures ...*treptococcus mitis* ATCC 903 the cell-...d character of both enzymes was indi-...d (4). For studies of the regulation of ...me synthesis, suitable disintegration meth... for the quantitation of enzyme activity ...ell extracts had to be evaluated. Disinte...ion methods are often adopted without ...r investigations into their effects on the ...erties of the enzymes under study. The ...ice of disintegration method for the pur...s of enzyme extraction has to be made ...an empirical basis. High enzyme yields ...y be obtained by methods with a high ef...ency of cellular disintegration which are ...ble to the particular enzyme.

The aim of the present investigation was to study and compare the disintegration efficiency of two fundamentally different methods and their effects on the yields and on the stability of hyaluronidase and aminopeptidase. The methods used were freeze-pressing according to *Edebo* (2) and autolysis *under* controlled conditions. Aminopeptidase was included in this investigation in order to permit comparisons between the extraction of two different enzymes and in order to evaluate the possibility of using aminopeptidase as an intracellular marker in this strain of *Streptococcus mitis*.

MATERIALS AND METHODS

Strain. Streptococcus mitis ATCC 903.

C...tivation technique and nutrient medium. Cul...ti...tions were performed under anaerobic conditions in ...stirred fermentor (TG 300, Biotec, Stockholm, Sweden) equipped with automatic pH-control. The nutrient medium and the cultivation technique have been described previously (4). In

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the biochemical tests

Film and spots	Tetrazolium reduction 0.009 % aerobic	0.009 % anaerobic	0.045 % aerobic	0.045 % anaerobic	Haemolysis	Haemag glutination	Haemad-sorption
3	3	23	4	30	β : 36	23	21
3	4	21	12	22	β 22	12	3
3	2	3	3	3	β : 3	0	0
11	2	9	4	11	α 1 β : 10	3	10
0	0	1	0	3	0	3	0
3	0	11	1	20	0	11	9
3	2	3	0	2	α 1 β : 1	0	0
2	2	2	1	1	β : 2	0	0
3	0	0	0	0	0	0	0

the five strains in this group were found to be identical. A one way cross reaction between strain H 756 and *M feliminutum* (BEN) was found both by the indirect immunofluorescence test and the growth inhibition test.

C The Anatomical Sources of Species and Serogroup

The anatomical sources of the isolated mycoplasma species and serogroup A are listed in Table 4

DISCUSSION

The glucose arginine and phosphatase tests are of great value in the biochemical grouping of strains isolated from dogs. On the basis of the reactions in these three tests it was possible to divide the strains into six groups. By the subsequent serological identification, no antigenic overlapping between different biochemical groups appeared to occur In addition to the tests mentioned haemolysis may be of some value Thus, all strains of *M anis M edwardii S ogroup A M cynos M ma ulosum* (except the type strain PG 15) and *M bovigen tal um* were found to be haemolytic, whereas *M p* *M gateae* and *M feliminutum* strains were non-haemolytic.

By the immunofluorescence examinations, the *M canis* strains were found to form a homogeneous group. In the growth inhibition experiments it seemed as if antiserum against one of the isolates was more reactive than the reference antiserum. Strains not inhibited under optimal growth conditions were inhibited when the test was performed under suboptimal conditions. This suggests that the growth inhibition test is made more sensitive by this modification (7)

Whether tested by immunofluorescence or growth inhibition, the *M edwardii* strains constituted a homogeneous serological group.

The serogroup A strains were found to form a homogeneous group. As they deviated from the established canine species they have to be further examined before a final classification can be made.

The characteristics of the strains belonging to *M cynos* have been described in detail elsewhere (11)

In the group of *M gateae* the strains belong to two serogroups (groups 1 and 2) re presented by strain H 372 and strain H 201 respectively This is most evident from the results of the metabolic inhibition test where

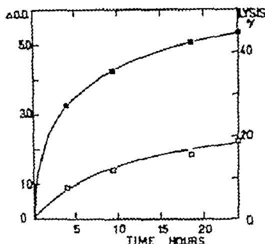


Fig 1 Kinetics of autolysis at 7 C and 37 C. Autolysis is expressed as the decrease in optical density (ΔO.D) and as per cent lysis in cell suspensions having an initial optical density of 12.0±0.1 The cells were suspended in 0.05 M phosphate buffer pH 6.8 supplemented with chloramphenicol (100 μg/ml) □——□ 7 C ■——■ 37° C.

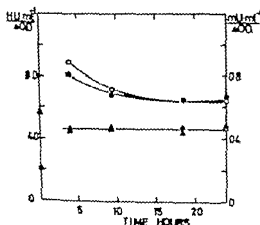


Fig 2 Effect of time and temperature on the specific hyaluronidase and aminopeptidase activity during autolysis. The specific activities of hyaluronidase and aminopeptidase during autolysis at 7 C and 37 C are plotted against incubation time In addition, specific enzyme activities in extracts prepared by freeze-pressing are plotted on the ordinate. Specific hyaluronidase activity during autolysis at 7 C ○——○ and at 37 C ●——● Specific hyaluronidase activity in extracts prepared by freeze-pressing ⊗ Specific aminopeptidase activity during autolysis at 7 C △——△ and at 37 C ▲——▲ Specific aminopeptidase activity in extracts prepared by freeze-pressing △

also seen that the degree of autolysis (ΔO.D) did not affect the specific activities within the range of values of ΔO.D of these experiments, shown in Fig 1 The incubation time did not influence the specific aminopeptidase activity while the specific hyaluronidase activity was higher in extracts incubated for less than 9 hours. On the ordinate of Fig 2 are also plotted the specific enzyme activities of extracts prepared by freeze-pressing The low specific hyaluronidase activity in extracts prepared by freeze pressing compared to that obtained by autolysis was found to be mainly due to the freezing and thawing procedures of the X press method. Almost the same loss in activity was found in experiments where cell-free extracts, obtained by autolysis, were subjected to freezing and thawing under the same temperature and time conditions as during freeze pressing The yield of specific aminopeptidase activity was high after freeze-pressing

The effect of time temperature and centrifugal force on isolated enzyme activity in extracts prepared by freeze-pressing Investigations on the stability of enzyme activity in extracts and in extracts containing cell debris is of major importance when the release of the enzyme is performed by autolysis for several hours and also when the enzyme preparations have to be stored before assay

The results of the experiments described above indicated that aminopeptidase activity was stable in cell extracts during autolysis. However the results were not conclusive regarding the stability of hyaluronidase during autolysis.

The isolation of bacterial enzymes from extracts is generally performed by centrifugation. Those proteins which are associated with such particulate matter as cell walls, cell-wall fragments, membranes and ribosomes are found in supernatants of low-speed centrifugations.

Experiments were performed in order to study the effect of freeze pressing on the activity and stability of hyaluronidase and aminopeptidase during incubation at 37 C and at 7 C of supernatants obtained by cen

TABLE 2. *Serological Examination of 118 Mycoplasma Strains Isolated from Dogs*

Number of strains in each biochemical group	Biochemical reaction			Antisera against	Number of strains positive in the serological tests	
	Glucose	Arginine	Phosphatase		Immuno-fluorescence	Growth inhibition
59	+	—	—	*M. canis* (PG 14)	35	9
				M. canis (H 11)	NT	27
				M. edwardii (PG 24)	21	21
				Serogroup A (H 542)	3	3
10	+	—	+	*M. cynos* (H 831)	10	NT
10	—	+	—	*M. gateae* (H 372) group 1	8	NT
				M. gateae (H 201) group 2	2	NT
32	—	+	+	*M. spumans* (PG 13) group I	14	30
				M. spumans (H 764) group II	16	
				M. maculosum (PG 15)	2	2
2	—	—	+	*M. bovigenitalium* (PG 11)	2	2
5	—	—	—	*M. feliminutum* (H 736)	5	NT

NT Not tested.

TABLE 3 *Serological Relationship between Strains of* Mycoplasma gateae

Antigens	Antisera against		
	H 372	H 201	*M. gateae* CS
Indirect immunofluorescence			
strain H 372 (Group 1)	80	<10	<10
strain H 201 (Group 2)	<10	60	80
M. gateae CS	80	60	80
Growth inhibition *			
strain H 372	5 mm	1 mm	1 mm
strain H 201	0 mm	4 mm	0 mm
M. gateae CS	0 mm	0 mm	4 mm
Metabolic inhibition			
strain H 372	128	0	0
strain H 201	8	≧ 2048	0
M. gateae CS	8	8	≧ 2048
Double immunodiffusion **			
strain H 201	a	ab	a

* The test was performed with B-medium containing 2.5 per cent horse serum and without yeast extract.

** Precipitating antigens are indicated by small letters.

only weak cross reactions occurred. According to the immunofluorescence study there is no cross reaction between strain H 372 and strain H 201 but both strains do cross react with the type strain *M. gateae* (CS). By growth inhibition examination there is a one-way cross between strain H 372 and strain H 201 and between strain H 372 and *M. gateae* but

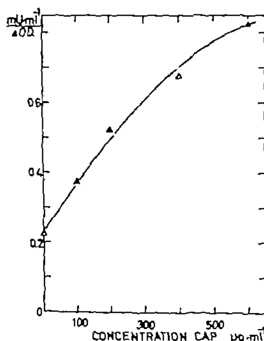


Fig. 6 Effect of chloramphenicol (CAP) on the activity of aminopeptidase in extracts prepared by autolysis. Autolysis was carried out at 7 C for 20 hours in 0.05 M phosphate buffer pH 6.8 containing different amounts of CAP. The specific aminopeptidase activity is plotted against concentration of CAP

ronidase synthesis. The formation of aminopeptidase, however, was not inhibited by CAP at concentrations of 100–400 µg/ml. In the present investigation it was observed that CAP increased the specific aminopeptidase activity of extracts prepared by autolysis. The following experiments were performed to study this effect. Autolysis was carried out at 7 C in buffer containing CAP at concentrations of 0–600 µg/ml. CAP had no significant effect on autolysis and did not affect the hyaluronidase activity of the extracts. The aminopeptidase activity of the extracts was increased by CAP in a *concentration dependent* manner as can be seen from Fig. 6. In this experiment the spectrophotometric determinations of aminopeptidase activity were made against controls containing the corresponding amount of CAP. These controls were treated like the test material. The controls were also measured against water at 578 nm. These measurements showed that CAP only very slightly affected the A_{578} readings. The effect of CAP on the aminopeptidase activity of cell-free extracts was investigated by adding CAP to final concentrations of 100–600 µg/ml to a cell-free extract prepared by autolysis in buffer not containing CAP. Aminopeptidase *activity of the extracts was not* affected by CAP at zero time nor after 20 h at 7 C.

Experiments were also carried out to study the effect of CAP on aminopeptidase activity in cell extracts prepared by freeze-pressing. Washed cells were suspended in buffer containing no CAP and crushed by the X-press method. After thawing, the suspension was centrifuged at 1 000 × *g* for 10 min. The supernatant was divided into two equal parts. To one part, CAP was added to a final concentration of 400 µg/ml, the other part was used as control. Both solutions were incubated at 7 C for 20 h and then centrifuged at 20 000 × *g* for 10 min. The aminopeptidase activity of the supernatant containing CAP was about 20 per cent higher than that of the *control*.

CAP had no effect on pH of the buffer in any of these experiments.

DISCUSSION

Several practical implications of the experiments on extraction of enzymes by autolysis and by freeze-pressing can be stated. Although freeze-pressing was found to be a more efficient method for *disintegration* of cells than autolysis, the latter method was found to be preferable to quantitate cell hyaluronidase and aminopeptidase strain. The efficiency of autolytic di tion could be increased by using buffers of high molarity. High aminopeptidase *could also* be freeze-pressing. In spite of the fact enzymes were successfully extracted lysis both at 37 C and at 7 C, at low temperature (7 C)

TABLE 4 *Anatomical Sources of Mycoplasma Species Isolated from Dogs*

	M spumans	*M canis*	*M maculosum*	*M edwardii*	*M cynos*	*M gateae*	*M feliminutum*	*M bovigenitalium*	*Serogroup A*
onjunctiva	1	1			1			1	
pper respiratory tract	18	25	2	16	3	4	1		3
ower respiratory tract	8			2	1		3	1	
male genital tract	1	7		2	2	1	1		
ale genital tract		2		1	3	5			

there is no cross reaction between strains H 201 and *M gateae* (CS) The finding of one common antigen in the double immunodiffusion test is in support of the assumption that strain H 372 and strain H 201 represent different serogroups within the species *M gateae*

Using the immunofluorescence method, the *M spumans* strains could be divided into two serogroups Group I with *M spumans* (PG 13) as representative strain and group II with strain H 764 as representative strain. In growth inhibition studies, it was not possible to differentiate between the serogroups and, as previously shown, it is not possible either on the basis of the gel electrophoresis pattern (12)

The isolated *M maculosum* and *M bovigenitalium* strains were serologically identical with the type strains *M maculosum* (PG 15) and *M bovigenitalium* (PG 11) respectively

Five strains were identified as *M feliminutum* as a representative strain (H 756) was found to give one way cross reaction with the type strain *M feliminutum* (BEN) whether tested by immunofluorescence or by growth inhibition.

The anatomical sources of the mycoplasma species *M spumans M canis M edwardii*, and *M cynos* seem to be both the respiratory and the genital tract. It should be mentioned that *M spumans* strains were recovered from the lung tissue of four dogs with pneumonia. *Edward & Fitzgerald* (4) isolated *M spumans* only from the vagina but it seems that this species also is a frequent finding in the respiratory tract.

Among the four mycoplasma strains isolated from conjunctiva, three were identified as *M spumans M canis* and *M cynos*

M maculosum has only been found in two cases and in both of these in the pharynx. This is a low frequency of isolation on the consideration that *M maculosum* colonies were identified by immunofluorescence in 22 out of 35 pharynx cultivations (13) The explanation may be that the cultivation conditions were not optimal for *M maculosum* for which reason the colonies remained small and were not often selected for cloning This hypothesis is partly confirmed by the fact that a *M maculosum* strain was cloned in one case from a pharynx cultivation in which only *M maculosum* colonies were identified by previous immunofluorescence.

M gateae has previously been reported as a feline species (8) but it appears from the present investigation that this species also occurs in the respiratory- and genital tract of dogs.

M feliminutum has previously been represented by one strain (*M feliminutum* BEN) isolated from the throat of a cat (8) In the present study five strains were isolated from dogs, in two cases from the lungs of dogs with pneumonia. Further studies are requested in order to determine whether *M feliminutum* may be a canine species rather than a feline

The isolation of *M bovigenitalium* from dogs has not been described previously In this study it was isolated from the conjunctiva and the lung of a dog with pneumonia.

The serogroup A strains were cultivated

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FORMATION AND RELEASE OF HYALURONIDASE AND AMINOPEPTIDASE IN GROWING CULTURES OF *STREPTOCOCCUS MITIS* ATCC 903

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The quantity of released and cell-bound hyaluronidase and aminopeptidase was determined in cultures of *Streptococcus mitis* grown under controlled conditions of pH, anaerobiosis, stirring and temperature. The results indicated that the formation of hyaluronidase was *completely repressed during exponential growth in the presence of glucose. Synthesis* of hyaluronidase occurred after the exhaustion of glucose during an autolytic phase. Aminopeptidase activity of the cells increased both in the presence of glucose and in the autolytic phase. Both enzymes were released into the medium by an autolytic process. Controlled autolysis is suggested as a physiologically useful release mechanism for intracellular enzymes directed at high molecular weight substrates which are unable to pass through the cytoplasmic membrane. Neither selective release of hyaluronidase from intact cells nor extracellular activity could be demonstrated.

Textbooks of microbiology often refer to hyaluronidase as an example of an extracellular enzyme in bacteria. This concept is based mainly on the investigations of Rogers (9 10). This author found that staphylococcal and streptococcal hyaluronidases were formed and liberated during active growth. The extracellularity of hyaluronidase is also appealing from a teleological point of view since the enzyme acts on a substrate of the order of a million in molecular weight which is unlikely to penetrate into the cell.

Peptidases, contrary to hyaluronidases, have been classified as truly intracellular enzymes in bacteria. The experimental evidence for an intracellular location of peptidases is well documented for different species (8). The results of previous studies of *Streptococcus mitis* aminopeptidase (3 4) are in agreement with an intracellular location of this enzyme.

In a previous article it has been shown that hyaluronidase and aminopeptidase of *Streptococcus mitis* were released into the growth medium during a phase of autolysis which followed the cessation of exponential growth (4). Cell-bound hyaluronidase and aminopeptidase were obtained in extracts after disintegration of cells by autolysis and by freeze-pressing (3).

The purpose of the present investigation was to study formation and release of hyaluronidase and aminopeptidase of *Streptococcus mitis* by quantitating and comparing cell-

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only from the upper respiratory tract Since this study was initiated another two strains identical with serogroup A were isolated The classification of this group will be given later

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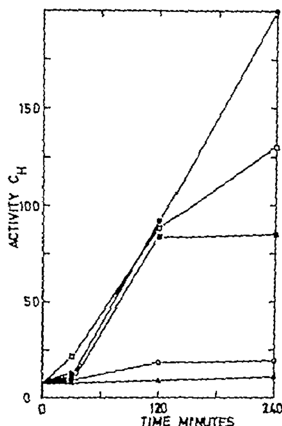


Fig. 7. Effect of proteose-peptones on the synthesis of hyaluronidase in non-growing cells. The specific hyaluronidase activity (C_H) was determined after incubation for various periods of time in proteose-peptone media containing various concentrations of proteose-peptone and in a casein hydrolysate medium. The incubation temperature was 37 C. Proteose-peptone concentrations (w/v): 8 per cent ●—●, 4 per cent □—□, 1 per cent ■—■, 0.1 per cent ○—○, Trypticase 4 per cent (w/v) △—△.

tent of the cells increased only very little in cells incubated in 0.1 per cent proteose-peptone. In contrast to hyaluronidase, the specific aminopeptidase activity increased in Trypticase medium at approximately the same rate as in the same concentration of proteose-peptone.

Effect of Peptones upon Autolysis

Neither autolysis occurring during incubation of cells in the peptone media nor autolysis during the subsequent incubation in phosphate buffer for extraction of the enzymes was affected by variations in the concentration of peptones.

DISCUSSION

The increasing hyaluronidase activity of the cells during incubation in proteose-peptone media reflects most probably "de novo" synthesis, as CAP was inhibitory. The lack of inhibitory effect of CAP and puromycin on the increase in aminopeptidase activity of the cells in proteose peptone media argues strongly against "de novo" synthesis of this enzyme under these conditions. The regulation of the aminopeptidase activity of the cells by activation or release of inhibition of the enzyme

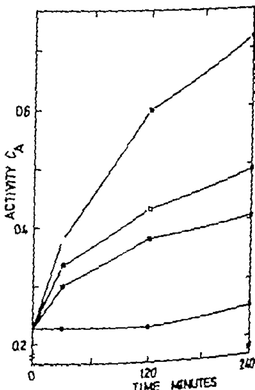


Fig. 8. Effect of proteose-peptone concentration on the increase in aminopeptidase activity of non-growing cells. The specific aminopeptidase activity was determined in cells incubated for various periods of time in proteose-peptone media containing various concentrations of proteose-peptone. The incubation temperature was 37 C. Proteose-peptone concentration (w/v): 8 per cent ●—●, 4 per cent □—□, 1 per cent ■—■, 0.1 per cent ○—○.

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ASSOCIATION OF CAPSID PROTEIN WITH SEMLIKI FOREST VIRUS MESSENGER RNAs

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Semliki Forest virus specific polysomes from infected HeLa cells were isolated. Part of the newly formed radioactively labelled protein remained associated with the messenger RNA after release of the nascent polypeptide chains with EDTA. The messenger RNA bound label consisted mostly of viral capsid protein. Direct isolation of ribonucleoproteins from the cytoplasm showed that the capsid protein binds to both 42 S and 26 S viral RNAs.

Semliki Forest virus (SFV), a group A arbovirus, is composed of a nucleocapsid which is surrounded by a lipoprotein envelope (13). The nucleocapsid consists of one RNA molecule, the 42 S RNA (mol.wt. 4 × 10⁶ 24, 27) and about 230 identical lysine-rich capsid proteins (mol.wt. 34 000) (2, 20, 23, 31). The envelope is composed of two virus-specific glycoproteins of similar size (mol.wt. 50,000) together with lipids from the host cell plasma membrane (22, 29, 33).

Virus-specific RNA and proteins are synthesized in the cytoplasm of the infected host cells (7, 9). The nucleocapsid is formed rapidly from 42 S RNA and capsid protein, whereafter the particle buds through the plasma membrane in which the envelope proteins are inserted (1, 7, 9, 32, 34).

Throughout the growth cycle two major RNAs are synthesized — the 42 S RNA which is the viral genome, and a 26 S species (mol. wt. 1.6 × 10⁶) (6, 11, 16, 24, 27, 37). Both are single-stranded but do not hybridize with each other (16), suggesting that the 26 S RNA is identical to part of the 42 S RNA as is the case for the closely related Sindbis virus (30). Small amounts of single-stranded 38 S, 33 S and 20 S RNAs are also formed (16, 24, 26).

The 42 S and 26 S RNAs are found in polysomes synthesizing viral proteins (19, 35). The 42/26 S RNA ratio in polysomes is the same independent of the labelling period (35). The 42 S RNA is withdrawn from messenger function by the continual formation of nucleocapsid, whereas the fate of 26 S RNA is not known. Here we report that the binding of capsid protein to both 42 S and 26 S RNA occurs in the polysomes.

MATERIALS AND METHODS

Viruses and cells. The origin and cultivation of Semliki Forest virus prototype strain (SFV) (17) and of HeLa cells have been previously described (34).

Isotope labelling. Monolayers of HeLa cells (1–3 × 10⁷) were infected with SFV at a multiplicity of infection of 50. After a one h absorption period at 37 °C the cells were washed 3 times with Hanks' salt solution and Eagles minimum essential medium (MEM) containing 0.2 % bovine serum albumin (BSA) and 2 µg/ml of actinomycin D was added. At the times indicated 100–300 µCi/10⁷ cells of ³H-uridine (25–30 Ci/mmole), in some cases together with 100 µg/ml of cycloheximide and/or 50–300 µCi/10⁷ cells of ³⁵S-methionine (20–30 Ci/mmole) in methionine-free MEM were given. ¹⁴C-labelled amino acids from Chlorella protein hydrolysate 20 µCi/10⁷ cells (54 mCi/milliatom of car

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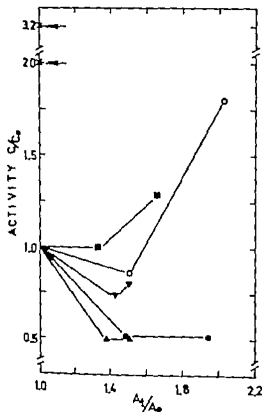


Fig 1 Effect of different carbohydrates on repression of hyaluronidase synthesis and on growth rate. Glucose-grown cells were incubated in aliquots in PP3 media containing various carbohydrates at a concentration of 0 17 M. Incubation times 30 min and 60 min. Incubation temperature 37 C. Hyaluronidase activity is expressed as the specific hyaluronidase activity (C) relative to the specific activity (C_o) of cells incubated in PP3 medium containing CAP. Relative growth is expressed as the quotients A_t/A_o at 30 min and 60 min.
▲——▲ mannose cultures, ●——● glucose cultures, ▼——▼ galactose cultures, ○——○ sucrose cultures, ■——■ fructose cultures.
The activity of cultures incubated in carbohydrate-free PP3 medium is indicated on the ordinate by arrows. The lower value represents the activity after incubation for 30 min and the upper value the activity after 60 min at 37 C.

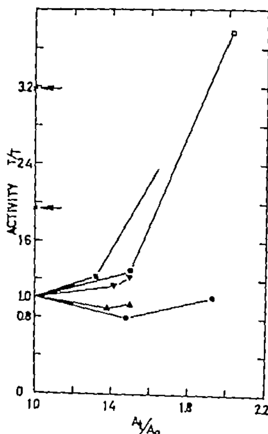


Fig 2 Effect of different carbohydrates on hyaluronidase repression. The activity is expressed as total hyaluronidase activity (T) of the culture relative to the total hyaluronidase activity (T) of the CAP-culture.
For symbols see Fig 1

RESULTS

It was shown earlier (5) that cells of *Streptococcus mitis* growing anaerobically under controlled conditions in PP1 medium had low level of hyaluronidase during late logarithmic growth. When these cells were transferred to carbohydrate-free proteose-peptone medium, synthesis of hyaluronidase started after a lag of about 10 min. CAP completely inhibited the synthesis of the enzyme under these conditions (6)

Effect of Various Carbohydrates on Hyaluronidase Synthesis and on Growth at 37° C

The influence of different carbohydrates, at a concentration of 0 17 M on the synthesis of hyaluronidase was investigated during incubation of the cells for 30 and 60 min at 37 C. Specific and total hyaluronidase activities relative to the corresponding activity of CAP incubated cells are plotted against relative growth in Figs. 1 and 2. Hyaluroni

bon) were used in the presence of 1/10 the normal concentration of amino acids in MEM as indicated. All isotopes were from The Radiochemical Centre, Amersham, England.

Cell fractionation Cells were harvested and cytoplasmic extracts prepared in 0.01 M NaCl 0.01 M Tris pH 7.4 0.0015 M $MgCl_2$ (RSB-Na) where after Triton X-100 was added to 1 per cent (w/v) as described previously (35) The cytoplasmic extracts were fractionated in 15–30 per cent (w/w) sucrose gradients (ribonuclease-free sucrose, Mann Research Laboratories, New York) in RSB-Na and centrifuged at 25,000 rev/min in a Spinco SW 27 rotor at 2 C as indicated. A part of each cytoplasmic extract was treated with 0.02–0.04 M EDTA and similarly fractionated in 0.01 M NaCl, 0.01 M Tris pH 7.4 0.01 M EDTA (Tris-EDTA buffer)

Analysis of RNA and proteins. RNA extracted with 2 per cent (w/v) sodium dodecyl sulphate (SDS) was analyzed in 15–30 per cent (w/w) sucrose gradients as described in (18) or by polyacrylamide-agarose gel electrophoresis as in (35) Analysis of proteins by electrophoresis in 5 per cent polyacrylamide gels (PAGE) containing SDS was as described in (34)

Other methods. ^{3}H-uridine labelled SFV was cultivated as described in (18) purified (36) and nucleocapsids and the 42 S RNA isolated from it by sucrose gradient centrifugation after treatment with Triton X 100 and SDS respectively (18) ^{32}P labelled 26 S RNA (carrier-free ^{32}P-orthophosphate from Institutt for Atomenergi, Kjeller Norway) was produced as in (16) Trichloracetic acid insoluble radioactivity was determined as in (17) Sedimentation constants were determined by the method of *Martin & Ames* (25) For determination of buoyant densities the samples were fixed with 5 per cent neutralized glutaraldehyde and centrifuged in preformed CsCl gradients (14) at 37,500 rev/min for 8–12 h in a Spinco SW 50 rotor

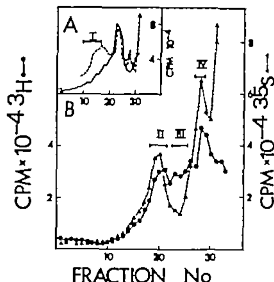


Fig 1 Polysomes and nucleoproteins from SFV infected HeLa cells. The cells were exposed to ^{3}H uridine (300 μCi/10^7 cells) at 5 h post infection in the presence of cycloheximide (100 μg/ml) At 5.30 h the inhibitor was removed by extensive washing and at 5.45 h ^{35}S-methionine (300 μCi/10^7 cells) was added. At 5.48 h post infection the cells were washed with ice-cold phosphate buffered saline harvested and homogenized in RSB-Na. Nuclei were pelleted by centrifugation at 250 × g for 5 min and Triton X 100 was added to the cytoplasm, thereafter part of the sample was analyzed on 15–30 per cent (w/w) sucrose gradient made in RSB-Na. Centrifugation at 25,000 rev/min for 2 h at 2 C in Spinco SW 27 rotor (A) To the other part of the cytoplasmic extract EDTA was added to 0.025 M and it was analyzed on a similar gradient made in Tris-EDTA buffer Centrifugation time 4 h (B) Acid insoluble radioactivity was determined from each 1 ml fraction. Bottom of gradient is at left. I–IV refer to pools which were made from the fractions indicated by the bars. (^{35}S —— ^{3}H)

RESULTS

When HeLa cells infected with Semliki Forest virus are pulse-labelled for 2–3 min with ^{35}S-methionine between 4 and 6 h post infection (p.i.) up to 45 per cent of the protein-bound label is found in viral polysomes. After a chase of for example, 3 min, 10–20 per cent is still associated with the polysomes. Immediately after the pulse the polysome-associated label is mostly in nascent polypeptide chains (as judged by the heterogeneous pattern in PAGE) but after the chase the capsid protein is greatly enriched in the polysome fraction (34) This finding suggested that the completed capsid protein remained associated with the viral messenger RNA.

In this study the association of virus-specific protein with newly formed RNA was investigated The RNA cannot be labelled with very short pulses of radioactive precursors, because of the slow equilibration of the nucleotide pools in eukaryotic cells. A 30 min pulse followed by 15 min incubation in the absence of the radioactive precursor was chosen for the labelling to be sure that most

that either mannose itself or some metabolite of mannose catabolism is an effector of hyaluronidase repression. The slow rate of utilization (β_{man}) of the effector keeps the concentration of the effector at a high level even at low values of α.

Fructose and metabolites of fructose catabolism did not seem to contribute to the effector pool although the rate of utilization of fructose and its intermediary metabolites was slow

The results of experiments with sucrose as growth substrate support the suggestion that fructose is not as effective as glucose (or mannose) in hyaluronidase repression.

Growth on glucose also produces effectors of hyaluronidase repression. However the high rate of utilization (β_{max}) brings the concentration of the effector to the level necessary for complete repression only at high values of α. This theory is based on the observation that glucose at high concentration gave a more intense repression than at low concentration and on the assumption that the rate of entry of glucose is dependent on the exogenous concentration.

It seems reasonable to assume that the effectors of hyaluronidase repression are closely related to glucose and that mannose which has great resemblance to glucose is also effective in producing effectors. Our results may be interpreted to agree with the theories that the intensity of the repression is proportional to the concentration of the effector (11)

In an investigation by *Mandelstam* (8) on repression of constitutive β-galactosidase in *E. coli* it was observed that the ability of a carbon source to support rapid growth was correlated to its ability to repress enzyme synthesis. No such correlation was observed in *Streptococcus mutis*. Sucrose supported growth at maximum rate, but reduced the rate of enzyme synthesis only for a limited period of growth. Mannose repressed enzyme synthesis completely for the whole experimental period although growth was slower than in glucose and sucrose.

Under complete repression of the synthesis of a cell-bound enzyme in a growing bacterial culture, the specific enzyme activity would be expected to "dilute out" and the total activity would be expected to remain constant if the enzyme is stable under the conditions of growth. The experimental data presented in Fig 1 indicate that the decrease in specific activity in mannose- and glucose grown cells was more rapid than that to be expected under conditions of complete repression of a stable enzyme. This phenomenon was also observed in experiments at 7 C (Fig 3) and in the experiments presented in Figs. 4 and 5 Although hyaluronidase has been found to be stable in cell-free extracts and in extracts containing cell debris (4) the results of the present experiments indicate that losses in activity occurred during growth.

Two effects of exogenous cyclic AMP on the synthesis of hyaluronidase were observed with this strain. Cyclic AMP partially inhibited the synthesis of hyaluronidase in nongrowing cells and cyclic AMP could partially relieve the repression exerted by glucose. The latter effect was not specific for cyclic AMP since 5 AMP could also partially relieve repression. These observations indicate that cyclic AMP may play a role in enzyme regulation in this strain and that this effect is different from the specific effect of cyclic AMP shown in gram-negative organisms (12) The slight increase in growth rate influenced by cyclic AMP (although not statistically significant) might have increased the rate of utilization of effector β so that the concentration of effectors reflected in the relation $\frac{\alpha}{\beta}$ was decreased. It is possible that even small changes in the concentration of effectors are reflected in the repression of enzyme synthesis.

Most investigations on repression of enzyme synthesis have been concerned with inducible enzymes such as those of the lactose system. The present study has permitted investigation of repression separate from induction by an exogenous inducer and the permeation of inducer into the cell.

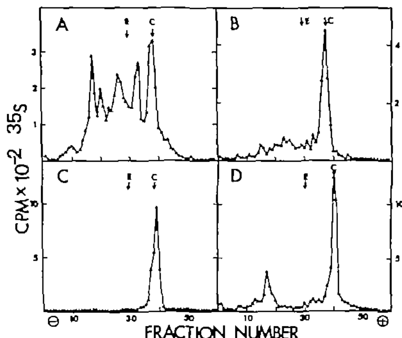


Fig 2 SDS-PAGE of newly formed protein from pools in Fig 1 A: pool I, B pool I treated with EDTA (0.025 M) layered on a cushion of 40 per cent (w/w) sucrose in Tris-EDTA buffer and centrifuged for 4 h at 45 000 rev/min in a SW 50.1 rotor t 2 C. The pellet was resuspended in electrophoresis buffer containing 1 per cent SDS and 1 per cent 2-mercaptoethanol. C pool II and D pool IV Viral envelope (E) and capsid (C) proteins were used as internal markers and their positions are shown by arrows.

of the radioactive RNA was released from the replication complex (16) Since during this period, a substantial amount of 42 S RNA could be consumed in the formation of nucleocapsids, the labelling of RNA was carried out in the presence of cycloheximide, which inhibits the synthesis of nucleocapsids (10 34)

SFV was grown in HeLa cells in the presence of actinomycin D (2 μg/ml) and the virus-specific RNA labelled with ^{3}H-uridine 5–5.30 h p i. with 100 μg/ml of cycloheximide in the medium. At 5.30 h p.i. the cycloheximide and ^{3}H-uridine were removed by washing with warm MEM, and the incubation continued for 15 min. This was necessary to allow full protein synthesising activity to be resumed. At 5 45 h p.i. ^{35}S-methionine was added. Three min later the cells were harvested and cytoplasmic extracts prepared in RSB-Na containing Triton X 100 (0 5 per cent) Part of the cytoplasmic extract was fractionated by centrifugation for 2 h in a 15–30 per cent sucrose gradient to obtain the polysome profile (Fig 1 A) The other part was treated with EDTA and sedimented for 4 h to resolve the viral ribonucleoproteins (RNP) (*Fig. 1 B*)

Polysomes

The distinct peak with a sedimentation value of 140 S (in Fig 1 A fraction 24) represents the viral nucleocapsid, since it contains only capsid protein and 42 S RNA and its density is 1 43 in the presence and absence of EDTA (Data not shown) The more rapidly sedimenting radioactivity (180–350 S, fr 1–18 in Fig. 1 A) represents the viral polysomes fulfilling our previously used criteria (35) their density is 1.50 g/cm^{3} before and 1 42–1 44 g/cm^{3} after treatment with 0.04 M EDTA treatment of the cells for 5 min

to investigate the relation between the presence of inhibitors against certain digestive enzymes and the content of feed particles, enzymes and bacteria in these intestines.

MATERIALS AND METHODS

Intestines. The material was randomly collected in connection with slaughtering of Norwegian Landrace pigs, between six and seven months of age (about 80 kg slaughter weight). The alimentary tracts, including the ventricle to the rectum were, after evisceration, immediately brought to the laboratory where the intestines were dissected from the mesenterium and laid out in their whole length on a table. The intestines were then—where specimens were to be taken out—cut longitudinally under aseptic conditions. Samples of the intestinal content were taken aseptically for enzymatic and bacteriological examinations. The amount and consistency of the content was recorded. Between 12 and 20 samples were taken from each small intestine, depending upon the variations of the contents. Samples were also taken from the ventricle and the large intestine. The samples were collected and examined within 1–2 hours after the pigs had been killed. (The animals were electrically stunned and killed by bleeding). Altogether 20 intestines have been investigated.

Bacteriological examination. Qualitative bacteriological investigation of the intestinal content was performed by using an inoculating loop to streak out the material on bromthymolblue-lactose agar plates (Nordic committee on food analysis 1969) and blood agar. Parallels of blood agar plates were incubated aerobically, anaerobically and in an atmosphere containing 10 per cent CO_2, and read after 24 and 48 hours. The bromthymolblue-lactose agar plates were incubated for 24 hours aerobically before reading. From some intestines preliminary studies with regard to the occurrence of Gram-negative, strictly anaerobic rods (Bacteroides) were carried out. For this purpose the method described by Fuller & Lev (1964) was used and samples were taken from the anterior and posterior end of the jejunum. Quantitative bacteriological investigation was mainly performed from parts of the intestines with relatively large amounts of content. Usually 4–5 samples from each intestine were examined in this way. The samples were diluted 10^{-} and 10 in saline, and 0.1 ml of the dilutions were transferred onto the surface of blood agar plates which were incubated as described above. For demonstration of bactericidal activity in the intestinal content an agar-diffusion method was used in which the principle was to put the content into wells in blood agar plates previously inoculated with one of the following test organisms *Sarcina lutea, Escherichia coli* of different origin, *Streptococcus* spp. and *Lactobacillus* spp.

The bacteria were classified into families, genera and in some cases into species on the basis of cultural, morphological and biochemical properties (Bergey's Manual 1957 *Edwards & Ewing* 1972)

Determination of enzyme activities. The enzymes investigated included proteinase, elastase, amylase and lipase. The qualitative and quantitative/semiquantitative determinations were performed by using the agar diffusion method as previously described for determination of proteinase (*Sandvik* 1962) elastase (*Merkara* 1964) amylase (*Harrigan & McCance* 1966) and lipase (*Ellinghausen jr & Sandvik* 1965)

The principle was to incorporate the relevant substrate, casein elastin, starch or tributyrin, into agar and then transfer 0.050 ml of each sample, into wells (7 mm in diameter) in the agar plates. After incubation, zones of precipitation (casein precipitation test or CP-test) as in the case of proteinase, or lysis zones as in the case of elastase and lipase, could be read directly or the zones indicating enzymatic activity could be read after developing with lugol as in the case of amylase activity. The diameters of the zones correlate with the enzyme concentration. For each intestine, the sample with the highest activity for the enzyme in question was diluted twofold before applying the dilutions into wells in the substrate-containing agar plate. The zone diameters were measured after a standard incubation time, and plotted against the dilution in order to give standard curves. By comparing the zone diameter caused by the various enzymes in each sample with the corresponding standard curve, a quantitative measure of the various enzyme activities was obtained. In the case of proteinase, the enzyme activity was also determined according to the Kunitz method (*Kunitz* 1947) For the determination of the presence and inhibitory spectrum of proteinase inhibitors, the crosswise casein precipitation inhibition test (CPI test, *Fossum* 1970 a) was used. To detect to which extent antiserum against swine trypsin inhibited the proteolytic activity in the different samples, the electrophoretic CPI-test was used (*Fossum* 1970 b)

Histological investigation. In some of the intestines certain areas of the mucosa were submitted to histological examination. The preparations were carried out according to ordinary histological techniques and stained with haematoxylin-eosin and van Gieson.

RESULTS

Intestinal content The ventricles contained various amounts of digested fodder Inspection of the small intestines revealed distinctly

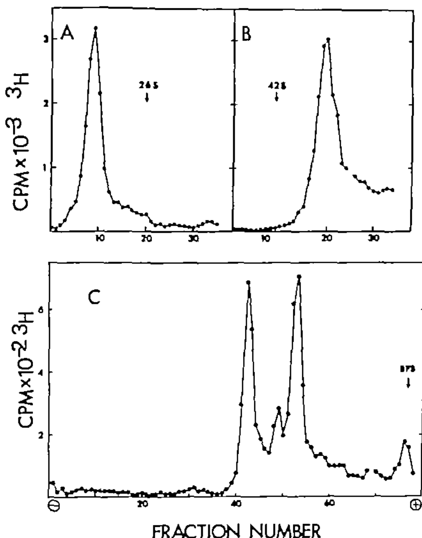


Fig 3 Analysis of RNAs from nucleoprotein pools shown in Fig 1 B. A pool II and B pool IV were sedimented in 15–30 per cent (w/w) sucrose gradients made in RSB-Na containing 0 1 per cent SDS centrifugation for 10 h at 4,000 rev/min in SW 27 rotor at 22 C. C pool III was analyzed by polyacrylamide-agarose (1 8 per cent and 0 5 per cent) gel electrophoresis in 0.6 × 70 cm gels at 10 V/cm, for 3 h. The radioactivity in 2 mm slices was determined. BPB = bromphenolblue.

with 100 μg/ml of puromycin prior to harvesting dissociates them and treatment of the cytoplasmic extract with EDTA (0 025 M) results in their disappearance (Fig 1 B)

The polypeptide pattern in PAGE from the polysomal fraction (pool I in Fig 1 A) is presented in Fig 2 A. Sucrose gradient analysis showed that both 42 S and 26 S RNAs were present in a label ratio of about 3.2 in the pool I (Data not shown) This ratio is similar to those observed in our previous experiments (35) By using cycloheximide the percentage of RNA associated with polysomes could be increased from 20 to 30 per cent of the total acid insoluble H activity This shows that the accumulation of messenger RNA can be achieved by inhibiting protein synthesis.

Ribonucleoproteins

The polysomes (pool I Fig 1 A) were treated with 0.025 M EDTA to dissociate

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THE ISOLATION OF *MYCOPLASMA PRIMATUM* DURING AN AUTOPSY STUDY OF THE MYCOPLASMA FLORA OF THE HUMAN URINARY TRACT

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The mycoplasmal flora of the urinary tract of 19 men and 21 women was investigated at autopsy Mycoplasmas were isolated from the urethra of 9 bodies. One of the isolates was identified biochemically and serologically as *M primatum*. This is the second isolation from man of this simian mycoplasma species. Three of the remaining isolates from the urethra were identified as *M hominis* one as *M fermentans* and four as *U urealyticum*. *U urealyticum* was isolated twice from the bladder urine. In one case, a strain of *U urealyticum* was isolated from the cortex of a kidney with histologically demonstrable non-specific inflammation.

Whereas the mycoplasmal flora of the human urethra and bladder has been the subject of numerous investigations (3 7 8, 10 15 17) only a few studies of the occurrence of mycoplasmas in the upper urinary tract have been reported (11 16, 17)

The purpose of the present work was to study the mycoplasmal flora of the urethra, bladder and upper urinary tract in an autopsy material.

MATERIAL AND METHODS

The investigation included unselected bodies of 19 men and 21 women. The average age was 72.3 year ranging from 8 to 89 years The kidneys were examined macroscopically and microscopically A diagnosis of acute pyelonephritis was made in one man and two women; one man and one woman showed chronic non-specific inflammatory lesions in the kidneys. Tumours were found in the kidneys of five, and arteriosclerosis in the kidneys of 28 bodies, while two had normal kidneys. Fifteen patients had received, within a month before death, antibiotics that are not inhibitory to mycoplasmas.

Received 13 74 Accepted 13 74

Requests for reprints should be addressed to A. C Thomsen The Institute of Medical Microbiology University of Aarhus, DK-8000 Aarhus C, Denmark.

Collection of Specimens

Specimens were collected during autopsy 6–30 hours post mortem. A pilot study including the bodies of 10 men and 10 women was carried out to compare the results of cultivation for mycoplasmas from the urethra immediately after death and at the time of autopsy Cultivation for *Ureaplasma urealyticum* at the two stages gave the same results, while the number of colony forming units (f u.) of large-colony mycoplasmas had slightly decreased at the time of autopsy

Specimens from the urethra were collected by rotating sterile cotton-tipped swabs in the external orificium. Following cauterization of the bladder surface, bladder urine was aspirated using sterile needle. The aspiration was successful in 15 cases only (10♂ and 5♀) an agonal emptying of the bladder preventing it in the remaining cases (9)

Specimens from the mucosal membrane of pelvis renis were taken by rotating sterile cotton-tipped

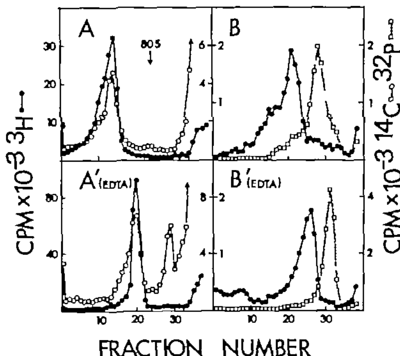


Fig 4 Sedimentation behaviour of viral and cytoplasmic nucleocapsids and isolated viral RNAs mixed with cytoplasm before and after treatment with EDTA A purified ^{3}H-uridine labelled SFV was mixed with cytoplasmic extract from infected HeLa cells labelled with ^{14}C-amino acids 5–6 h post infection (20 μCi/10^7 cells) Before centrifugation Triton X 100 was added to 1 per cent. B isolated ^{3}H-uridine labelled 42 S and ^{32}P labelled 26 S RNAs mixed with triton treated HeLa cell cytoplasm. Centrifugation in 15–30 per cent (w/w) sucrose gradients in RSB-N for 4 h at 25,000 rev/min in SW 27 rotor at 2° C. The samples treated with EDTA (0.04 M) were analyzed in gradients made in Tris-EDTA buffer

the ribosomes releasing the nascent polypeptides and RNPs isolated by pelleting them through a cushion of 40 per cent (w/w) sucrose in Tris-EDTA buffer The pellet was dissolved in electrophoresis buffer containing SDS (1 per cent) and 2-mercaptoethanol (1 per cent) and the proteins were analyzed by PAGE (Fig. 2 B) A protein co-migrating with the viral capsid protein marker formed the major peak, which represented 50–60 per cent of the total radioactivity recovered from the gel. Similar analysis carried out from the TCA precipitated supernatant fraction showed the pattern of nascent chains

Sedimentation of the EDTA treated cytoplasm revealed two distinct peaks of newly synthesized protein (Fig 1 B) These peaks, which sedimented at 100 S (pool II) and 60 S (pool IV) cosedimented with RNA label. Analysis of the proteins by PAGE showed that a polypeptide indistinguishable from capsid protein, was associated with both the 100 S and 60 S RNP peaks and with the material between them (Fig 2 C, D) In the 60 S peak a large protein with an apparent molecular weight of about 110,000 was also present. Only 42 S RNA was found in the 100 S peak whereas the 60 S peak contained 26 S. Both these RNAs and some 33 S RNA were found in the intermediate region (Fig 3)

Nucleocapsid

Only a small amount of radioactivity sedimented in the position of the 140 S nucleocapsid after EDTA treatment of the cytoplasm (fractions 12–15 in Fig. 1 B) This indicates that EDTA treatment had reduced

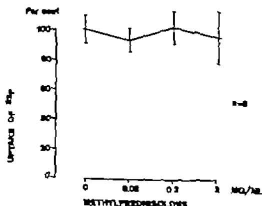


Fig 5 Phagocytosis of ^{32}P-labelled *E. coli* by PMN in the presence of 10 per cent serum. Influence of pre-incubating PMN with *methylprednisolone* at 37° C for 30 min. The rate of uptake of label without methylprednisolone is set as 100 per cent. I = ± 1 SD (n = number of observations)

tion of PMN in methylprednisolone at concentrations up to 2 mg per ml did not significantly reduce the rate of uptake ($p > 0.10$)

Pre-incubation of Methylprednisolone with Bacteria

At two different temperatures (0 and 37 C) *E. coli*, suspended in KRG were pre-incubated for 20 *min* with or without 2 mg methylprednisolone per *ml*. After centrifugation and washing in KRG the bacteria were resuspended and incubated in KRG with 10 per cent serum for 15 min at 37 C. As shown in Fig. 6, the rate of release of label from bacteria was not reduced when methylprednisolone was added to the pre-incubation medium.

The Effect of Different Steroid Compounds

The effect on the bactericidal activity of serum is illustrated in Fig 7 The release of label induced by serum was reduced to the same degree by the succinate and phosphate compound of hydrocortisone ($p > 0.10$) as well as by methylprednisolone succinate ($p > 0.10$) in equimolar concentrations of hormones (2 mg per ml)

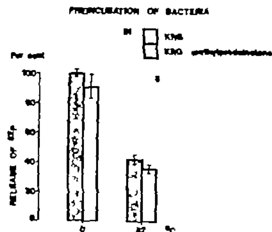


Fig 6. Pre-incubation of ^{32}P-labelled *E. coli* in KRG with or without methylprednisolone (2 mg per ml) for 20 min at 0 and 37° C. Influence on release of label from bacteria in the presence of 10 per cent serum. The rate of release from bacteria pre-incubated at 0° C with methylprednisolone is set as 100 per cent. I = ± 1 SD (n = *number of observations*)

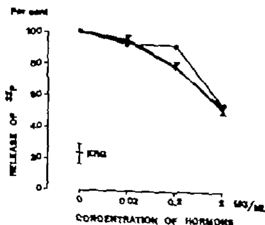


Fig 7 Effect of different steroid compounds on the bactericidal activity of serum towards ^{32}P-labelled *E. coli* after incubation of 37 C for 15 min. The rate of release of label from bacteria in the presence of serum without steroid drugs is set as 100 per cent.

the sedimentation rate of the newly formed nucleocapsid. This was confirmed using nucleocapsid isolated from purified virus. In this experiment SFV infected HeLa cells were labelled with ^{14}C-amino acids 5–6 h p.i. to allow the accumulation of radioactive nucleocapsids (28). Virus nucleocapsid labelled with ^{3}H uridine was mixed with the cytoplasm of the ^{14}C labelled cells and analyzed by sucrose gradient centrifugation (Fig. 4 A).

In the absence of EDTA the ^{14}C label cosedimented with the ^{3}H labelled virus-derived nucleocapsid at 140 S. It is notable that only small amounts of ^{14}C activity sedimented between 40 S and 120 S. In the EDTA treated sample (Fig. 4 A) the sedimentation rate of the viral nucleocapsid was reduced to about 100 S but the density was not changed (35) which indicates that the RNA:protein ratio remains unaltered. Thus the reduced S-value must be due to a configurational change of the nucleocapsid.

Most of the ^{14}C-amino acid radioactivity cosedimented with the viral nucleocapsid at 100 S following EDTA treatment which suggests a similar alteration in the configuration of the cytoplasmic nucleocapsid. EDTA treatment revealed another peak, which sedimented at 60 S at the position of the 26 S RNA nucleoprotein. The origin of this peak was not studied further.

Nucleoproteins Formed with Exogenous RNA

To exclude the possibility that RNPs from the polysomes would be aggregation products of cytoplasmic, non-messenger RNAs and capsid protein the following experiment was performed. 26 S RNA from SFV infected cells labelled with ^{32}P-orthophosphate and 42 S RNA from ^{3}H uridine labelled SFV were isolated by sucrose gradient centrifugation. The RNAs were mixed with the cytoplasm from infected HeLa cells and analyzed on sucrose gradients in the absence and presence of EDTA (Fig. 4 B, 4 B'). The 42 S RNA sedimented as a relatively broad peak with a maximum at 95 S and 26 S RNA more sharply at about 60 S. Very small amounts sedimented at the position of the polysomes (> 180 S). EDTA affected the sedimentation of these artificial RNP complexes so that 42 S and 26 S RNAs sedimented now at 70 S and 40 S respectively. This result suggests that RNPs synthesized in SFV infected cells are different from those formed artificially.

DISCUSSION

We have previously shown that in SFV infected cells radioactively labelled RNA sedimenting faster than 180 S is associated almost exclusively with polysomes synthesizing viral proteins (35). Both the density and sedimentation characteristics of these polysomes are changed after treatment with EDTA indicating that they do not contain viral nucleocapsid, the density of which is unaltered by EDTA (35). Here we report that capsid protein, which is the only virus-specific protein with a molecular weight of 34,000 (8, 12, 28), was isolated from polysomes after the ribosomes had been removed. In other words the newly formed capsid protein is directly bound to the viral messenger RNA, whereas the other viral polypeptides are not. This explains why capsid protein is enriched in the polysomes in pulse-chase experiments with radioactive amino acids (34).

Friedman and Grimley have shown (10) that no soluble capsid protein is found after labelling for 60 min. We have recently demonstrated that this is true even after a 2 min pulse. The possibility that the capsid protein is bound to membranous structures was excluded by using Triton X 100 (34). Thus it seems that the capsid protein is quantitatively bound to RNA immediately after being synthesized.

When the protein synthesis proceeds the amount of capsid protein bound to a given RNA molecule should increase. One would expect that as this process continues a maximum amount of capsid protein is finally bound to this messenger molecule. When protein synthesis was inhibited with cycloheximide, and hence the binding of capsid protein to RNA also prevented the amount

induction after confrontation with host tissue antigens? 3) if so, can the cells producing the inhibiting factors be enriched by passage through an anti Ig coated column? 4) does the allogeneic host milieu inhibit the development of immature T cells into mature T cells?

As shown in Table 2, the spleens of mice transplanted with allogeneic bone marrow cells contained significantly higher numbers of θ positive cells than their syngeneic counterparts. Thus, the allogeneic host milieu does not suppress the development of θ positive cells from allogeneic stem cells. That the thus developed θ positive cells were not derived from the minor population of θ positive cells present in normal bone marrow was shown by pretreatment of the allogeneic bone marrow cells with anti-θ antiserum plus complement (Tables 2 and 4). This fact also invalidates the questions 2) and 3) viz. that mature T cells from the bone marrow upon their confrontation with host tissue antigens, may produce substances which inhibit the development of θ positive cells from stem cells.

Our results concerning the functional activity of θ positive cells in the present system have shown that transplantation of bone marrow cells to allogeneic mice does not enable the cells from such mice to mediate a specific or even a third party GVH reaction (Tables 4 5 6, 8). Allogeneic irradiation chimaeras have been shown to display a depressed helper function in humoral antibody production (6) and a prolonged skin graft survival (12). Thus, there seems to be a discrepancy between the relatively high level of θ positive cells in allogeneic irradiation chimaeras and their immunological capability. Therefore we tested the possibility that these θ positive cells were immunologically immature T cells. This was done using a collaborative GVH assay in which immature and mature T cells have been shown to interact synergistically (2, 8). Our efforts were unsuccessful, however (Table 8).

The present results and previous work from other laboratories (5 6 12) leave us with the puzzling problem what is the reason for the immunologically inertness of the θ positive cells in allogeneic irradiation chimaeras? A provocative explanation would be the elimination or neutralization of precursor T cells reactive against certain histocompatibility antigens causes a simultaneous and general depression of the T cell functions in such animals. An alternative explanation is that the development of immunologically mature T cells from immature T cells is dependent on a fully reconstituted and normally functioning haematopoietic system (5). The former hypothesis is tested at present by experiments where bone marrow cells are adsorbed onto fibroblast monolayers of relevant or irrelevant histocompatibility type prior to transplantation into irradiated syngeneic and allogeneic mice.

The skilful technical assistance of Miss *Birgit Rasmussen* and Miss *Birgit Hertel Wulff* is gratefully acknowledged. The authors also wish to thank Mr *Harry Andersen*, Institute for Experimental Immunology University of Copenhagen for irradiating the mice and Mr *Egon Christensen* and Mr *Flemming Velsted* for injection and bleeding of the mice.

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of RNA in the polysomes was clearly enriched following the removal of the inhibition. One explanation for this observation is that the "capsid protein saturated RNA" becomes inactive as a messenger. If so the capsid protein would regulate the turnover rate of the viral RNAs in their messenger function.

Studies of SFV nucleocapsid formation by pulse-chase experiments, in which amino acid label was used, showed that all the radioactive capsid protein is formed within 2–3 min after the end of the pulse whereas the maximum radioactivity in the 140 S nucleocapsid is obtained after a 5–10 min chase (34). This indicates that unlabelled capsid protein is added to a labelled nucleocapsid precursor resulting in the maturation of the 140 S structure during the chase period. That continuous protein synthesis is needed for the maturation of the nucleocapsid is shown by the use of cycloheximide (10 34).

The experimental evidence presented above supports the idea that the messenger 42 S RNA-capsid protein complex actually is this nucleocapsid precursor. Thus the primary reaction in the nucleocapsid assembly occurs between the capsid protein and RNA. We assume that after the addition of a sufficient amount of protein to the RNA the ribonucleoprotein begins to fold, finally becoming the spherical nucleocapsid particle.

Previous work in this laboratory has shown that a substantial proportion of the RNA can be released from the nucleocapsid by ribonuclease. After digestion the particle has a reduced S value and a lower density (18). In the process the nucleocapsid diameter decreases from 39 to 32 nm (4). This strongly suggests that the RNA plays an important structural role in the nucleocapsid. No da[illegible] concerning protein-protein interactions [illegible] structure are so far available. Howev[illegible] formation of interprotein bonds is p[illegible] necessary for a folding process.

The assembly of the helical [illegible] virus (TMV) is controlled b[illegible] titrating carboxyl-carbox[illegible] about 7) which preven[illegible] protein subunits befor[illegible] RNA in a favourable way (5). In the absence of RNA aggregation of protein subunits into a helix does take place at a pH of about 6 indicating that either RNA or H ions can trigger the helix formation (21). Similar carboxyl-carboxylate pairs are responsible for the reversible pH dependent size change of some small isometric plant viruses (3 15). Since the SFV nucleocapsid undergoes an irreversible contraction from 38 to 32 nm at pH 5.6–6.2 (36) it is tempting to speculate that a similar type of mechanism triggers the folding of the ribonucleoprotein into the spherical particle.

The significance of the binding of capsid protein to the 26 S RNA remains open. We could not demonstrate accumulation of 26 S RNA nucleoprotein comparable to the nucleocapsid without the use of EDTA, which suggests a different turnover mechanism for this RNA.

The expert technical assistance of Miss *Ritva Saarlema* and Mrs *Marja Langer* is gratefully acknowledged. The investigation was supported by grants from *Sigrid Jusélius Foundation*, *The National Research Council for Medical Sciences* and *The Association of Finnish Life Assurance Companies*, Helsinki, Finland. Actinomycin D was a gift from *Merck Sharp & Dohme*.

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DISCUSSION

The stable L-phase variants of bacteria were found to have a greater susceptibility to lysolecithin than the mycoplasmas previously examined (9). This differential sensitivity may provide a novel criterion for distinguishing between L-phase variants and mycoplasmas.

Lysolecithin seems to have an important part in the killing of mycoplasmas by tissue extracts (4, 10). Because L-phase variants are highly susceptible to lysolecithin it is plausible that it may also interfere with their recovery from clinical specimens. The lethal effect of tissue extracts on mycoplasmas may be overcome in different ways (10) and it seems likely that similar approaches may be of value in isolating L-phase variants. The reasons why mycoplasmas, L-phase variants and their parent bacteria are different in their susceptibility to lysolecithin are not clear but the amount of cholesterol incorporated in the cell membranes or walls may be important. In this respect, mycoplasmas, the membranes of which are known to contain cholesterol in contrast to those of acholeplasmas (1), are more susceptible than acholeplasmas to lysolecithin (9). Further more cholesterol is incorporated into the cell membranes of stable L-phase variants of certain bacteria than into the walls of their parent bacteria (12). This might explain, at least in part, why we have found stable L-phase variants to be more susceptible to lysolecithin than the corresponding bacteria. However differences in the cholesterol content of cell membranes are not likely to be the whole explanation for variations in their susceptibility to lysolecithin. L-phase variants of *Streptococcus pyogenes* and *Staphylococcus aureus* contain only slightly more cholesterol than the parent bacteria (12) and yet we found variants of these species to be *much* more susceptible to lysolecithin than the corresponding bacteria.

Hartsock et al. (5) found that bacteria, mycoplasmas and L-phase variants in the order mentioned had a decreasing susceptibility to streptococcal bacteriocin which they attributed to the relative increase of cholesterol in these organisms. In our experiments, although L-phase variants were more susceptible than mycoplasmas, only certain bacterial species were less susceptible than mycoplasmas. Indeed, pronounced differences in susceptibility to lysolecithin were noted with the bacteria tested. It is notable that the anaerobes were the most susceptible. Further studies are required to establish whether such a susceptibility might be one factor contributing to the difficulties of isolating anaerobic bacteria from clinical specimens. The reasons for the different lysolecithin sensitivities are unknown. Certainly there was no correlation between lysolecithin sensitivity and the gram-staining property of the bacteria and it seems unlikely that cholesterol in the cell wall is an important factor.

The toxic effect of lysolecithin on tissue-culture cells makes it difficult to interpret the susceptibility of the viruses themselves. Even if the tissue-culture cells appeared visually normal they might have been damaged and less capable of supporting virus multiplication. However it is also possible that the differences in the susceptibility of viruses to lysolecithin reflect differences in the composition of the virus envelopes. The presence of lipid in influenzavirus and its absence in rhinoviruses may explain why the former was sensitive to lysolecithin but not the latter. The apparent insensitivity of adenovirus to lysolecithin might also be due to the absence of lipid in this virus. However it was only possible to test it *against a low* concentration of lysolecithin because of the relatively high susceptibility of the cells used in this experiment. It is worth considering whether tissue homogenates kill ether-sensitive viruses and if so whether this might be due to the formation of lysolecithin.

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TABLE 4. *Similarity Matrix* [cut off]
Staphylococcus *Strains Expressed by a Transfer* [cut off]

Species/Strain§		1		3	4	5	6	7	8	9	10	11	12
1 *M luteus*	210	×											
2. *M luteus*	337	94	×										
3 *M l teus*	732	84	82	×									
4 *M luteus*	1335	73	85	87	×								
5 *M luteus*	2266	88	91	91	80	×							
6. *M* sp.	810	90	87	83	69	93	×						
7 *M varians*	268	90	90	88	69	89	87	×					
8. *M mucilaginosus*	2417	93	90	82	71	90	81	88	×				
9 *M mucilaginosus*	2392	84	80	70	69	87	78	80	88	×			
10. *M m ilaginosus*	2393	90	85	76	68	90	85	85	96	96	×		
11 *M mucilaginosus*	2486	89	87	81	74	89	81	65	97	95	98	×	
12. *M mucilaginosus*	4239/68	86	86	83	75	87	73	82	93	91	97	97	×
13. *M roseus*	679	85	82	82	69	82	93	77	79	68	73	74	73
14 *M roseus*	557	86	86	88	74	80	86	81	82	74	78	79	77
15. *M roseus*	837	71	71	66	59	66	67	59	55	48	51	50	48
16. *M roseus*	2179	84	86	84	76	77	79	73	74	62	68	68	70
17 *M roseus*	2257	82	84	84	75	77	79	74	70	60	65	66	63
18. *S aureus*	885	70	69	75	59	78	77	73	74	61	67	68	69
19 *S aureus*	1484	63	64	64	52	68	80	66	61	50	55	54	53
20. *S aureus*	2022	66	66	67	52	69	73	68	62	54	58	59	53
21 *S aureus*	2301	72	72	78	64	74	82	76	76	63	69	70	70
22. *S aureus*	2351	73	71	74	60	72	82	73	74	6?	68	68	67
23. *S epidermidis*	2124	62	62	68	55	66	76	66	64	51	57	58	58
24 *S epidermidis*	50	61	61	66	52	64	74	64	62	49	55	55	55
25 *S epidermidis*	2446	58	58	63	49	62	74	62	60	47	52	52	52
26. *S epidermidis*	2210	72	75	81	65	78	84	73	75	62	68	68	69
27 *S epidermidis*	2340	77	78	82	70	80	88	78	78	66	73	73	72
28. *S* sp.	2429	74	73	76	63	77	88	73	72	61	67	66	65
29 *S saprophyticus*	883	64	64	68	55	69	81	69	60	50	55	54	54

* See text for definition and explanation.

§ See Table 1 for the complete strain designations.

gram of the fatty acid composition for all strains. The micrococci and staphylococci form two distinct clusters fusing only at the 65 per cent level. Within the genus *Micrococcus* the species *M roseus* and *M mucilaginosus* each forms two distinct clusters. The former is fully clustered at a lower level than the latter. The "*M luteus-group*" in the phenogram contains both the *M* sp., and the *M varians* (see also Table 5). One of the *M luteus* strains (no. 4) even joins its peers only after the "*M luteus*-group" has fused with the *M mucilaginosus* cluster. Within the genus *Staphylococcus* the preconceived species are not distinctly separated. It is notable that two of the *S aureus* strains cluster with *two of the S epidermidis strains* which were both coagulase and *mannitol* negative.

DISCUSSION

Interpretation of Fatty Acid Composition

Methyl branched fatty acids, distinctive of many Gram-positive bacteria (14 35) were found to be the dominant class of fatty acids of all strains examined. This is in accordance with previous studies of fatty acid composition of the *Micrococcaceae* (7 10 20 31 32, 33). The considerable qualitative and quantitative inter laboratory differences are probably due to the variations in culture conditions and the techniques employed.

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RAPID DIAGNOSIS BY IMMUNOFLUORESCENCE OF VIRAL INFECTIONS ASSOCIATED WITH THE CROUP SYNDROME IN CHILDREN

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Diagnostic attempts by indirect immunofluorescence (IF) were made for parainfluenza-, respiratory syncytial (RS) and adenovirus on laryngo-pharyngeal cells aspirated within a few hours of admission from 87 patients with a diagnosis of croup. Parainfluenza virus antigen was demonstrated in samples of secretion cells from 33 out of 44 patients from whom parainfluenza virus was isolated in tissue culture. Furthermore, in six patients parainfluenza virus infection was demonstrated serologically. RS-virus was never isolated. One diagnosis of RS-virus infection made by IF was confirmed by the complement fixation (CF) test. Adenovirus was isolated in eight cases, none of which were diagnosed by IF in spite of one showing titre increase in the CF-test. Paired sera were available from 32 of the 87 patients. No influenza or *Mycoplasma pneumoniae* infections could be diagnosed by the CF-test. The IF-technique appears to be an important supplement to the rapid diagnosis of virus infections in patients suffering from croup.

In recent years several workers have published results of investigations where immunofluorescent techniques have been employed for the diagnosis of respiratory diseases in infants and children. It has been well established that parainfluenza virus infections, which play a major part in the croup syndrome of children (4), can be diagnosed by this method (7, 13). Respiratory syncytial (RS) virus as well as adenovirus are occasionally associated with croup (4) and reports on their identification by immunofluorescence (IF) have appeared during the last few years (3, 6, 10, 15, 16, 18, 20, 8, 14).

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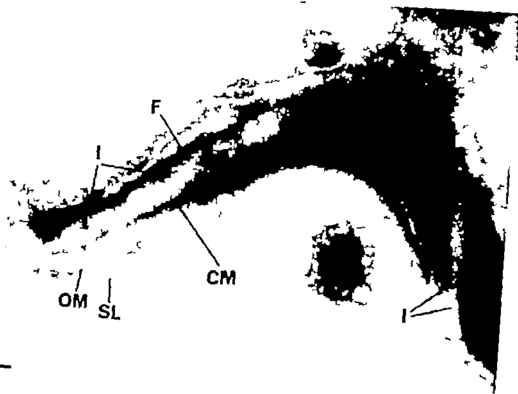
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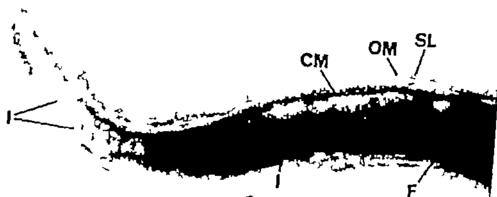
In this paper the simultaneous application of the indirect IF for parainfluenza-, RS- and adenovirus is presented. Cells from laryngo-pharyngeal secretions from a group of patients hospitalized with a diagnosis of croup have been employed.

MATERIALS AND METHODS

1) *Collection and Preparation of Pharyngo-laryngeal Secretions*

The collection and f rther preparation of specimens for inoculation into tissue culture and examination by IF were done as described previously by other investigators (3, 6, 20). In short, pharyngo-laryngeal secretions were aspirated within a few hours after the patient had been admitted to hospital; the specimens were brought without delay to the laboratory supplemented with transport medium consisting of Hanks' balanced salt solution


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(BSS) with 1 per cent bovine serumalbumin and antibiotics. At the laboratory the specimens were centrifuged at 1 000 rev/min for 5 min. The deposit was resuspended and washed 2–3 times in Hanks BSS. Finally the cells were resuspended in a small volume of Hanks BBS and transferred to plastic-sprayed slides, prepared as described by *Goldman* (9). After air-drying the cells were fixed in acetone for 10 min at room temperature. An adequate volume of the same cell suspension was left for isolation of virus in tissue culture.

2) *Isolation and Identification of Virus*

Cells from pharyngo-laryngeal secretions washed and resuspended in Hanks' BBS—as described above—were inoculated into each of three tissue culture tubes after the addition of 100 I U penicillin 100 μg streptomycin and 5 I U Fungizone® per ml. The tissue cultures used were HEp-2, primary African green monkey kidney cells (MK) and primary human foetal fibroblast (HFF). HEp-2 cells were maintained in Eagle's MEM with 2 per cent foetal bovine serum. MK-cells were maintained in Parker 199 without serum, and HFF-cells in enriched Eagle's (12) with 2 per cent foetal bovine serum.

a) *Parainfluenza virus.* Parainfluenza virus was isolated in MK-cultures, haemadsorption tests being carried out on all MK-tubes at 5, 10 and 15 days after inoculation. Parainfluenza virus was identified by the haemadsorption-inhibition test, using commercially (Microbiological Associates, Inc., Maryland, U.S.A.) available parainfluenza type 1, 2 and 3 antisera (2).

b) *Respiratory syncytial virus.* Attempts to isolate RS-virus were made in HEp-2 cell cultures which were observed for a fortnight following inoculation of the specimen.

c) *Adenovirus.* When the characteristic cytopathetic effect (CPE) of adenovirus degeneration was observed in the inoculated tissue culture tubes, a CF-test was performed on the antigen present, using a pair of human sera known to show a more than four-fold titre rise against adenovirus antigen. No attempt was made to determine the type of adenovirus involved.

3) *Direct Examination of Clinical Specimen employing Indirect Immunofluorescence*

a) *Preparation of antisera.* Antisera against parainfluenza type 1, 2 and 3 virus and RS-virus were prepared in 300 g female guinea-pigs by intranasal instillation of infectious virus of tissue culture origin (the parainfluenza virus strains were kindly supplied by Colindale Laboratories, London, and the RS-virus was the established American Long strain). Serum from each animal was screened for CF-antibody against the virus in question prior to the immunization. All the virus strains employed, except parainfluenza type 1 virus, were grown in VERO-cells maintained in Parker's medium 199 without serum. Parainfluenza type 1 virus was grown in MK-cells also maintained in Parker's medium 199 without serum. Before intranasal instillation of approximately 10^7 $TCID_{50}$ of the appropriate virus strain the animal was anaesthetised by intraperitoneal injection of sodium thiomebumal (Leopental® Leo) 5 mg/100 g body weight. By this method, a deep anaesthesia, lasting for 1–2 hours, was achieved, during which the virus established itself in the naso-pharyngeal epithelium of the animal. This procedure was repeated after one week, to ensure that all animals were infected. One week later the animals were tested for CF-antibodies against homologous and heterologous virus strains. All animals, except those who had received harvest from uninfected tissue culture cells for production of negative control sera, showed a high titre of homologous CF-antibody and were accordingly exsanguinated together with the control animals. The resulting sera were tested in the CF test and sera with high homologous titres as well as the negative control sera were pooled and used for IF.

Antiserum against adenovirus group antigen was prepared, as described by *Spence et al.* (19) (adenovirus type 2 was kindly supplied by Dr. *I. R. Pedersen*, Institute of Medical Microbiology, Copenhagen). According to this method (19) purified hexon antigen was obtained from tissue cultures infected with adenovirus. The antigen was tested in the CF-test against the above mentioned human antisera, and showed a titre of 1:16 against the convalescent serum. Female 300 g guinea-pigs were immunized by intradermal injection of this antigen in combination with Freund's complete adjuvant. The injection was repeated twice at intervals of one week with antigen without adjuvant. Two weeks after the final injection the animals were exsanguinated, and the sera were tested for CF-antibody to adenovirus antigen. The sera with the highest titres were pooled and used for IF. To ensure the specificity of the antigen-antiserum system, a crossed immunoelectrophoresis with hexon antigen against the antiserum was performed. Only one line of precipitate was formed, which indicated the purity of the system. Furthermore it was demonstrated by immunoelectrophoresis that the antiserum reacted with antigens prepared from tissue cultures infected with several different types of adenovirus.

Finally all antisera mentioned were purified to obtain pure IgG and IgA, following the method described by *Harboe & Ingild* (11).

Anti-guinea-pig globulin was prepared in rabbit by intradermal injection of 100 μl of a solution of guinea-pig IgG and IgA prepared from a pool of normal guinea-pig sera and purified as mentioned

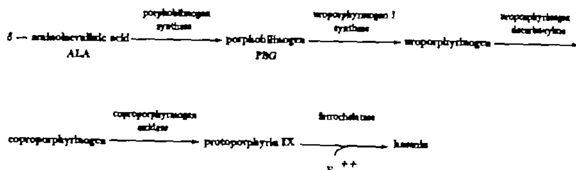


Fig 1 The main steps in the biosynthesis of porphyrins and haemin (see review by *Granick & Mauzerall* 1961)

a perfect correlation between absence of haemin requirement and the ability to convert ALA to porphyrins. Free porphyrins and other intermediates in the haemin biosynthetic pathway were excreted by strains of haemin independent *Haemophilus* species, as they are by many other bacteria if kept under conditions in which formation of cytocromes is limited (*Lascelles* 1956) The present paper describes a simple and rapid routine test for porphyrin synthesis based on the findings.

MATERIALS AND METHODS

Bacterial strains 134 *Haemophilus* strains were used for the study 89 of these strains were isolated from the upper respiratory tract of children (*Kilian et al.* 1972) The remaining 45 named strains listed in Table 1 were received from culture collections.

Growth medium Bacteria were grown on chocolate agar plates heated 10 per cent defibrinated horse blood in Blood Agar Base (Difco) The plates were incubated in air plus 10 per cent CO_2 at 37 C for 24 to 48 hours. For procedures including semi-quantitative estimation of synthesized porphobilinogen the bacteria were harvested, centrifuged (3000 *g* for 10 min) and washed once in 0.85 per cent (w/v) NaCl and finally suspended in the test fluid to give 1.5 .5 mg dry weight per ml. Dry weight was estimated after drying at 110 C to constant weight For the standard routine procedure applied to all strains, a loopfull of bacteria (about 1 mg) were suspended directly in 0.5 ml of the enzyme substrate contained in a Widal tube

The enzyme substrate for the porphyrin test was as described by *Lascelles* (1956) δ aminolaevulinic acid hydrocloride (Sigma) 2 mM and $MgSO_4$ 0.8 mM in 0.1 M phosphate buffer (Sørensen pH 6.9 The reaction mixtures were incubated at 37 C. A suspension of the bacterial cells in the test fluid without ALA acted as a control.

Determination of porphobilinogen and porphyrins Synthesized porphobilinogen was determined after incubation by adding one volume of *Kovács* (1928) modification of Ehrlich's reagent (p-dimethylaminobenzaldehyde 5 *g* amylalcohol 75 ml and conc. HCl 25 ml) After vigorous shaking the mixture was left for a few minutes to permit separation of the water and alcoholic phases. A red colour in the lower water phase is in this system indicative of PBG (see for ex. *Schwartz et al.* 1960) Semi-quantitative estimation of the PBG in the water phase was done at 560 nm on a Spectronic 20 photometer (*Bausch & Lomb*) after removal of the bacteria by centrifugation (5000 *g* for 10 min) For the demonstration of porphyrins their well-known red fluorescence in Wood's light was employed, using a Philips HPW 125 W lamp with maximum emmision at 360 nm.

Method for the determination of haemin requirement For comparative purposes the haemin requirement of the strains was determined by serial transfers on two defined agar plate media "XV" and "V" as described previously (*Kilian et al.* 1972)

RESULTS

A total of 50 strains were able to convert ALA to PBG and further on to porphyrins as revealed by an always simultaneous positive reaction of both substances. The fluorescence due to porphyrins was exhibited from the bacterial sediment as well as from the supernatants, but often it was first visible in the former The remaining 84 strains produced neither PBG nor porphyrins. The results applying to the named strains are listed in Table 1

above. Initially these globulins were injected in combination with Freund's complete adjuvant. The injection was then repeated three times at intervals of a fortnight. The rabbits were bled one week later and sera collected. Pure IgG and IgA were obtained as mentioned earlier. The amounts of antibody to guinea-pig globulins (IgG and IgA) present were determined in passive gel diffusion tests. Antisera showing distinct precipitates in high dilutions were pooled and used for conjugation with fluorescein isothiocyanate (FITC).

The conjugation procedures were very similar to those described by *The & Feltkamp* (21) and, after the initial conjugation they consisted in purification steps on a Sephadex G-25 column followed by ion-exchanging on a DEAE-Sephadex A 50 column. Both columns were equilibrated with PBS (pH 7.2, 0.15 NaCl). The first column was eluted with PBS, whereas the second column was eluted with a continuous phosphate-buffered NaCl-gradient in the range of 0.15–0.6 M. The fluorescein-protein (F/P) ratio in each fraction collected was photometrically determined, as described by *The & Feltkamp* (21). Only fractions with $0.8 < E^{1}_{495}: E^{1}_{280} < 0.95$ were pooled. The conjugate prepared by this method showed mol F/P = 3.5 corresponding to protein concentration of 4 mg/ml and a FITC-concentration of 0.035 mg/ml.

All antisera and the conjugate were absorbed to uninfected human- and monkey tissue culture cells. The absorptions were carried out at 37 °C for one hour using 5–10 mill. cells/ml antiserum. The suspensions were then centrifuged at 10 000 × G for 20 min, and the supernatants were dialysed for 24 hours against PBS containing 1 mM sodium-azide. Antisera and conjugate were then stored at +4 °C. The corresponding negative control sera were treated in the same way.

b) *Staining procedures.* Slides containing spots of washed and fixed pharyngo-laryngeal cells from the same patient were stained with antisera against parainfluenza type 1, 2 and 3 –, RS- and adenovirus by adding one drop of antiserum to each spot in the upper row and one drop of corresponding negative control serum to each spot in the lower row. Control slides containing virus-infected and uninfected tissue culture cells were run in parallel. Optimal dilutions of antisera and conjugate for IF were initially determined on the same control slides. It was found that a high multiplicity of infection and an early harvest employing versene improved the specific viral fluorescence of the infected control cells. Fixation of control cells took place in acetone for 10 min at room temperature. Antigens and antisera reacted for 30 min at 37 °C in a moist chamber. The slides were then washed twice with PBS for 10 min and once with deionized water using magnetic stirring. After air drying at 37 °C, the slides were stained with the FITC-conjugate by adding one drop to each cell spot, followed by incubation at 37 °C for 30 min. The washing procedures were repeated and the slides were dried and mounted in 10 per cent Elvanol® in tris-HCl-buffer pH 7.6. The slides were examined in a Leitz microscope with dark field illumination, using a tungsten lamp equipped with an interference filter as primary filter and an absorbing glass filtre as secondary filter.

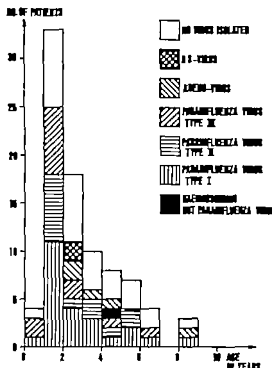


Fig. 1. Virus infections diagnosed by virus isolation and/or CF-tests in 87 patients with a diagnosis of croup. In double infections with RS- and adenovirus, only the coexisting para-influenza virus is recorded in the figure.

4) *Group of Patients Studied*

Pharyngo-laryngeal secretions were collected from 87 children who were admitted to the ear-nose- and throat department of the Glostrup hospital, Copenhagen, with a diagnosis of croup during the period from May 1972 to January 1973. The age distribution of the patients is shown in Fig. 1. More than 50 per cent of the patients were below the age of three. 59 were boys and 28 were girls.

5) *CF-tests Performed on the Patients' Sera*

Acute and convalescent sera were obtained from 52 (60 per cent) of the 87 patients. CF-tests, using para-influenza type 1, 2 and 3 –, RS-, adeno- and influenza type A, B and C virus antigens and

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	May	June	Aug	Sept.	Oct.	Nov	Dec	Jan.	Total
Parainfluenza I	–	2	2	10	5	4	1	–	24
Parainfluenza II	1	–	3	5	2	1	1	–	13
Parainfluenza III	–	1	3	1	4	3	1	1	14
Adeno	–	2	–	–		1	–	2	5
RS	–	–	–	–	–	1	1	–	2
–	3	4	4	7	1	5	3	2	29
Total	4	9	12	23	12	15	7	5	87

* July 1972 is excluded from the table as the collection of specimens was stopped in this month. In the double-infections with parainfluenza and RS-virus or adenovirus, only the coexisting parainfluenza virus is recorded.

Mycoplasma pneumoniae antigen, were performed on the sera, according to the Microtiter® method, with 0.025 ml volumes and 2.5 units of complement with overnight fixation at 4° C. The antigens for the parainfluenza-, RS- and adenovirus were prepared in tissue cultures, according to standard procedures (17). Influenza type A, B and C virus antigens were of commercial origin (Microbiological Associates, Inc., Maryland U.S.A.) and mycoplasma antigen was kindly supplied by Dr. *A. Lind* Statens Seruminstitut, Copenhagen. Control sera were of commercial origin (Microbiological Associates, Inc., Maryland U.S.A.) except for adenovirus- and mycoplasma antiserum. Adenovirus antiserum was the previously mentioned human sera kindly supplied by Dr. *Carl Mordhorst* Statens Seruminstitut, Copenhagen, and mycoplasma antiserum was also kindly supplied by Dr. *K. Lind*.

RESULTS

As appears from Fig. 1, the number of patients in the different age groups studied is too small to show any difference with respect to the virus types involved in the disease. An accumulation of croup admissions in the autumn is demonstrated by Table 1, while the percentage of the specimens revealing virus seems to be rather constant during the entire period.

Antisera used for IF were preliminarily screened in the CF test, followed by determination of the optimal dilution to be employed for IF. The results are recorded in Table 2. The antiguinea-pig FITC-conjugate was used diluted 1:5. The type of fluorescence observed with parainfluenza virus and RS-virus in virusinfected tissue culture cells as well as in cells from pharyngo-laryngeal secretions had the same configuration as that described earlier by other investigators (6, 7). In cells from pharyngo-laryngeal secretions, the appearance of at least two of these very typical cells in a specimen (i.e. one spot of cells on the slide) was required for the establishment of a positive diagnosis by IF. Adenovirus revealed a granular fluorescence, restricted firmly to the nucleus within tissue culture cells infected with adenovirus type 2, 20 hours before staining was performed.

The results of diagnostic attempts by IF are compared with virus isolation in Table 3. The number of false negative results found

TABLE 2 *Reciprocal Complement Fixation Titres and Immunofluorescent Titres of Guinea-pig Hyperimmune Sera Used for Identification of Virus in Pharyngo-laryngeal Secretion Cells from Children with a Diagnosis of Croup*

Virus type	CF-titres	IF-titres
Parainfluenza I	256	50
Parainfluenza II	256	25
Parainfluenza III	512	25
RS	256	50
Adeno	512	80

TABLE 3 *Comparison of the Immunofluorescent T hnique and Virus Isolation Applied to Specimens from Patients with   Diagnosis of Croup*

[illegible]	[illegible]		[illegible]		TOTAL
	[illegible]	[illegible]	[illegible]	[illegible]	
PARAINFLUENZA I	[illegible]	1 1)	0	[illegible]	[illegible]
II	[illegible]	1	0	[illegible]	[illegible]
III	7	3	1	[illegible]	11 [illegible]
RS-[illegible]	1	0	1 2)	[illegible]	1 [illegible]
[illegible] 4)	1	1 3)	0	[illegible]	[illegible]
[illegible]	1	1	0	[illegible]	1 [illegible]
[illegible]	[illegible]	[illegible]	0	[illegible]	[illegible]
[illegible]	[illegible]	17	2	[illegible]	[illegible]

1) Three of these were isolated but lost before identification however sera from the same patients showed titre rise to parainfluenza type I in CF-tests. In addition, sera from one patient in this group were negative in the CF test with parainfluenza antigen (cf. Table 4)
2) Sera from this patient showed titre rise to RS-virus antigen (cf. Table 4)
3) Sera were present from three of these patients a titre rise to adenovirus antigen was demonstrable only in one instance.
4) Furthermore adenovirus was on three occasions isolated together with parainfluenza virus.

co-positivity: 33/50 (66 %)
co-negativity: 35/37 (95 %)
overall agreement: 68/87 (78 %)
after *Buck & Gart* (1)

by IF for the parainfluenza virus infection was mainly due to an insufficient number of intact cells from the patients. Only one false positive IF-diagnosis was made, as the RS-virus infection diagnosed was confirmed by CF test (cf. Table 4) As regards adenovirus, at least one false negative IF-diagnosis seems to be present in a patient from whom adenovirus was isolated and from whom sera showed titre increase to adenovirus antigen (cf Table 4) On the three occasions of isolation of adenovirus together with parainfluenza virus, no titre increase to adenovirus was found, whereas increases of parainfluenza CF-antibodies were demon trated The isola tion of adenovirus is therefor   nsidered unimportant in relation to the parainfluenza virus isolated in these cases. The haemadsorbing virus isolated in MK-cells was neither inhibited by parainfluenza typing sera, nor could any titre rise be demonstrated in sera from the same patient (cf Tables 3 and 4)

Table 4 records the results of three different diagnostic procedures applied to specimens from the 52 patients from whom acute and convalescent sera could be obtained. Although these sera were tested against influenza virus type A, B and C and against *Mycoplasma pneumoniae* antigen as well, the results of these tests are not included in the table because only negative results were achieved with these antigens. As appears from the table, CF tests seem to identify seven infections more than the isolation attempts reveal. The double infections with RS- and parainfluenza virus revealed by CF tests, are believed to represent successive infections with these agents. None of the adenovirus infections that could be investigated in CF tests, except one, showed titre rise to adenovirus antigen. The adenovirus infections diagnosed are therefore probably of a latent nature, and this is consistent with the negative IF results obtained with these infections (cf Tables 3 and 4)

DISCUSSION AND CONCLUSION

Although antisera against parainfluenza and RS-virus were produced by *infection* of guinea-pigs, it was found that absorption of these antisera to uninfected tissue culture cells improved the IF This may indicate that intranasal instillation of undiluted tissue culture material provoked some immunity to cellular products. The purification of antisera to obtain pure IgG and IgA furthermore improved specific IF It is believed that this is due to the fact that only proteins of antibody type are present in these antisera, whereas albumin and lipoproteins, which may attribute to unspecific staining have been removed It should be stressed that this purification step can be performed practically

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adenovirus infections by IF directly on upper respiratory cells from patients few papers have appeared and the results seem rather disappointing (8 14)

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"PITTING" AND "CORROSION" OF THE SURFACE OF AGAR CULTURES BY COLONIES OF SOME BACTERIA FROM THE RESPIRATORY TRACT

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A large number of bacterial strains from the upper respiratory tract were examined for production of imprints of the colonies on blood agar plates. *Moraxella* strains regularly grew with corroding colonies and most cultures produced spreading zones around the colonies after prolonged incubation. *Neisseria (Moraxella Branhamella) catarrhalis* and *N. pharyngis* colonies produced marked corrosion without spreading zones. *Micrococcus mucilaginosus*, *Staphylococcus aureus* and *S. epidermidis* and some of the strains of *Streptococcus pneumoniae* and *N. meningitidis* produced slight to moderate depressions in the agar with or without moderate corrosion. About 10 per cent of the strains of alpha- and beta-haemolytic streptococci failed to produce imprints on the agar. Of the remaining strains the majority produced slight to moderate depressions with or without "corrosion". About 12 per cent of the strains produced colonies which were deeply imbedded or infiltrated in the agar. The possibility of an association of these phenomena with other physiological characters, as in some *Moraxella* species, is discussed.

"Pitting" and "corrosion", i.e. production of imprints in the surface of agar cultures by bacterial colonies, has been described for several bacterial species: *Eikenella* (*Bacteroides*) *corrodens* (5, 8, 10, 12), *Moraxella kingii* (9), *M. nonliquefaciens* (11), *M. bovis* (16), α-haemolytic streptococci (9), but generally little attention has been paid to such phenomena, and the effect of bacterial colonies on agar surfaces is rarely mentioned in the literature.

The fact that the "corrosion" phenomenon in certain species has been found to be firmly associated with fimbriation (2, 6), twitching motility (7), competence in genetic transformation (3), and capacity for colonization of mucous membranes (17) suggested that it might be worth while to study the effect of colonies of some other bacteria on agar surfaces.

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MATERIALS AND METHODS

Bacterial Strains

The strains to be studied were isolated from 5 per cent human blood agar plate cultures of nasal and pharyngeal swabs. The cultures were incubated at 35–37° C overnight. One or (in the case of α-haemolytic streptococci) two colonies of the organism to be studied were picked with a loop and spread on fresh blood agar plates so as to obtain well separated colonies. The secondary plates were incubated at 35° C in a humid athmosphere for 48 h, after which some colonies were scraped off the surface with a platinum loop, as gently as possible in order to avoid making marks in the agar

with the loop. The agar surface was then scrutinized with the naked eye and with a magnifying glass for marks made in the agar by the colonies. Cultures of some species (*Moraxell* *Neisseri*) were returned to the incubator and observed for several days for signs of spreading of the colonies

The following organisms were selected for study *Moraxella* species, *Neisseria* spp. *Micrococcus mucilaginosus* α-haemolytic streptococci, β-haemolytic streptococci *Staphylococcus aureus* *S* *pidermidis* and *Str ptococcus pneumoniae*

Identification Methods

Moraxella Identified by microscopy and the oxidase test with dimethyl-p-phenylendiamine. The species was not determined, but it may be assumed (1) that the majority of strains were *M* *nonliquefaciens*

Neisseria. Identified by microscopy oxidase test, test for acid production from glucose, maltose and saccharose, and, in the case of meningococcus-like colonies, lactose. Strains growing with opaque colonies and failing to attack sugars were considered to be *N* (or *Mor xella* or *B anhamella*) *catarrhalis* Strains with shiny translucent colonies (usually with characteristic smell) acidifying glucose and maltose, but not saccharose or lactose, were identified as *N* *meningitidis* and strains with opaque colonies, fermenting glucose, maltose and saccharose were lumped together under the designation *N* *pharyngis*.

M *mucilaginosus* Identified by microscopy and appearance of the colonies.

Staphylococcus. Identified by ppearance of colonies, microscopy and the coagulase test Strains giving a positive coagulase test (and usually with yellow colonies) were identified as *S* *aureus* and strains with negative coagulase test (and usually white colonies) as *S* *pidermidis*. The possibility that some of the latter strains may have been *Micrococcus* cannot be excluded.

Alpha-haemolytic streptococci Identified by colony appearance, microscopy and by production of α-haemolysis.

Beta-haemolytic streptococci Identified by appearance of colonies, by microscopy and by production of β-haemolysis (if in doubt confirmed by haemolysis of blood broth) The serological group was not determined.

S *pneumoniae* Apperance of the colonies, microscopy and production of haemolysis (mostly α) If in doubt, confirmed by test for sensitivity to optochin.

Grading of Re ding

The readings were roughly graded from — to + + — no sign of imprint in the aga was detected. + imprints in the aga were visible, ranging from a just perceptible smooth mark with slight depression to clearly visible pits, with more or less distinct corrosion e. depressions in the agar in the form of a peripheral groove and/or a central pit. + + : The colony had infiltrated more or less completely into the agar. The colonies often appeared to hav sunk slightly down into the agar and were surrounded by shallow grooves. In some cases it was possible to remove the colonies with some force, leaving deep pits with torn surfaces In other cases the colonies were so firmly embedded in the agar that they could only be removed by cutting out the agar where they were located. Of the colonies showing + + reactions, little more than one half showed the latter type of reaction.

RESULTS

A summary of the results is shown in Table 1 It appears that some degree of imprint or corrosion of the agar surface is extremely common in these bacteria which live on the mucous membranes of the upper respiratory tract. Some comments on the individual species or groups are required.

Moraxella. Only one out of 40 strains failed to produce pits in the agar The possibility that this strain may have belonged to a different species than the others cannot be excluded. In all the other strains corrosion was rather marked with a distinct groove in the agar corresponding to the periphery of the colony as reported before (2, 11) When the cultures were left in the incubator for several days, as many as 25 showed some colonies surrounded by films of spreading growth. Some of the strains examined in the early phase of the study may not have been incubated long enough to detect this phenomenon, and the figure 25 probably is too low The number of spreading colonies in the individual cultures varied from one or two to the majority of colonies.

N *catarrhalis* Sixty of the strains were isolated from the nose and three from the pharynx. Among the total number of *Neisseria* strains from the nose 60 were *N* *catarrhalis* seven *N* *meningitidis* and only a single strain was classified as *N* *pharyngis* thus confirming the findings by *Bøvre* (1) All except two strains showed marked pitting, usually with a distinct groove corresponding to

TABLE 1 *Pitting and Corrosion of Agar by Colonies of Bacteria from the Respiratory Tract*

Organism	Degree of corrosion			Total number of strains
	—	+	+ +	
Moraxella	1	39	0	40
N catarrhalis	2	61	0	63
N meningitidis	4	6	0	10
N pharyngis	0	24	0	24
S aureus	0	11	0	11
S epidermidis	0	25	0	25
α-haemolytic streptococci	32	257	41	330
β-haemolytic streptococci	3	13	4	20
S pneumoniae	5	5	0	10
M mucilaginosus	0	23	0	23

— no imprint of the colonies.
+ slight to moderate depression with or without corrosion.
+ + colonies deeply embedded or infiltrated in the agar

the periphery of the colony and a small central conical pit

None of the strains of this species, nor of *N meningitidis* and *N pharyngis* showed spreading zones after prolonged incubation.

N meningitidis Six out of 10 strains showed shallow smooth depressions under the colonies, without signs of corrosion. Due to the soft consistency of the colonies, it was difficult to remove them completely without leaving a film of growth that might have obscured possible depressions.

N pharyngis All 24 strains showed corrosion, usually of an appearance similar to that in *N catarrhalis* but in most cases with slightly less marked depressions, i.e. a shallower peripheral groove. Among the strains of gramnegative diplococci from the pharynx, 23 were *N pharyngis* one *N meningitidis* and three *N catarrhalis*

Staphylococci. All strains of *S aureus* and *S epidermidis* showed some degree of pitting, varying from a superficial smooth slight depression without any corrosion to a more marked, but only moderate depression with a narrow and shallow peripheral furrow

M mucilaginosus. These organisms are characterized by viscous, more or less strongly mucoid colonies with a tendency to adhere firmly to the agar in some cases so firmly that it was nearly impossible to remove the colonies without damaging the agar All of 23 strains showed easily visible depressions under the colonies, without marked corrosion.

Alpha-haemolytic streptococci. The strains showed great variations. In 32 strains (c. 10 per cent) no marks on the agar were seen. In 257 strains there were clearly visible marks under the colonies, varying from just perceptible smooth even marks with only very slight depression to marked depressions with a central pit and/or a peripheral groove.

Forty-one strains (c. 12.4 per cent) were so firmly embedded in the agar that the colonies could only be forcibly removed, leaving deep pits with torn surfaces (17 strains) or they could not be removed at all without cutting out the agar where they were located (24 strains) When these cultures were inspected, the colonies appeared to have sunk slightly down into the agar and the tops of the colonies were surrounded by shallow grooves

Beta haemolytic streptococci These strains showed similar variation as the α-haemolytic strains. Three out of 20 strains failed to show any marks in the agar 13 strains showed moderate pitting, with or without moderate corrosion, and the colonies of four strains were deeply embedded in the agar and could only be removed with difficulty

The serogroups of these strains were not

determined. Some of them were only represented by a very few colonies in the primary cultures, whereas others occurred as the predominating organism in the culture and obviously were the cause of infections.

S pneumoniae Among 10 strains only five produced very slight marks in the agar but, as in the case of the meningococci, it was difficult to remove the colonies without leaving a film of growth which might have hidden marks in the agar

DISCUSSION

The results show that the colonies of the great majority of examined strains produced some sort of imprint on the agar plates. The effect varied from just perceptible, smooth hardly depressed marks, through marked depressions with distinct pits or grooves to complete infiltration of the agar

The nature and significance of these different types of imprint on the agar may not be the same, but it should be possible, and might be worth while, to try to find out which of them are associated with other physiological traits, such as fimbriation, adhesiveness, motility competence in genetic transformation and capacity for parasitism.

It has now been well-established that the spreading-corroding colony type of *M nonliquefaciens* (and other *Moraxella* species) the SC-type is associated with fimbriation, twitching motility and competence in transformation (2 3 7) Whether fimbriation also is a prerequisite for capacity to colonize mucous membranes, is not known, but the fact that nearly all freshly isolated strains show this type of colony is highly suggestive and may perhaps indicate that some kind of selective pressure acts in favour of this colony type on the mucous membranes. The experiences obtained with *M bovis* (17) and with *N gonorrhoeae* (13 14 18, 19 20) suggest a relationship between fimbriation and ability to adhere to and grow on mucous membranes and to cause disease. The corrosion patterns produced by *N catarrhalis* and *N pharyngis* are similar although the colonies of these species do not spread like those of *M nonliquefaciens* The fact that nearly all freshly isolated strains of these species grow with corroding colonies, and that fimbriation (21) and competence in transformation have been demonstrated in both species, suggests the possibility of an association of characteristics similar to that in *M nonliquefaciens* and *N gonorrhoeae* It remains to be seen whether an unfimbriated and noncorroding stage exists in these species.

In this connection it may be mentioned that gonococcus colonies in primary cultures from pathological material, known mainly to be type 1 and type 2 colonies (13 14 15) produce marked pitting of the agar surface (unpublished observations) It may also be recalled that all strains of *N elongata* studied so far (4) have grown with the SC-type of colony and the strains which have been tested for this, have been competent in transformation.

The significance of the smooth marks of slight depression in the agar produced by staphylococci, *M mucilaginosus* and by some strains of *S pneumoniae* and *N meningitidis* is obscure, and further studies are needed to clarify the association of these phenomena, if any with other physiological characters.

The α- and β haemolytic streptococci show some special features. In about 10 per cent of the strains imprints were not detected. Most strains produced moderate depressions with or without grooves or pits in the agar and about 12 per cent of the strains were deeply embedded in the agar It would seem to be of considerable interest to study the association of these phenomena with other characteristics.

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SEARCH FOR THYMIDINE PHOSPHORYLASE, NUCLEOSIDE DEOXYRIBOSYLTRANSFERASE AND THYMIDINE KINASE IN GENUS *NEISSERIA*

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The enzymes thymidine phosphorylase, nucleoside deoxyribosyltransferase and thymidine kinase were searched for in species of *Neisseria*. Activities corresponding to these enzymes could not be found in the species examined.

It has previously been found that *Neisseria meningitidis* lacks the enzymes known to be involved in the utilization of exogenous thymine or thymidine for incorporation into DNA (13). These enzymes are thymidine phosphorylase EC 2.4.2.4, nucleoside deoxyribosyltransferase EC 2.4.2.6 and thymidine kinase EC 2.7.1.21 (1).

In the present work several species within the genus *Neisseria* have been examined for the presence of these enzyme functions to see if the situation is the same as in *N. meningitidis* (13). Also the species *N. catarrhalis*, *N. ovis* and *N. caviae* are included. These three species are, on the basis of genetic data, proposed to be removed from the genus *Neisseria* (5, 9), and it was of interest to find out if these "false neisseriae" differ enzymatically from the other species.

MATERIALS AND METHODS

Neisseria strains. The strains of the different *Neisseria* species are listed in Table 1. All the strains were from the same sources as listed by *Holten* (11). The strains were characterized culturally and biochemically by *Holten* (11) according to *Berger* (3) and *Bøvre* (4).

Cell free extract. Overnight cultures on blood agar medium incubated at 37° C in humid atmosphere with increased CO_2 content, were used for preparing cell free extracts for the enzyme assays. Approximately 0.1 g cells, wet weight, were suspended in 1 ml 0.05 M Tris/HCl buffer at pH 7.6 before ultrasonic disintegration (13). Unless otherwise noted the crude extracts were used immediately for the thymidine kinase assay. The same extracts which had been kept frozen at −20° C for 1 to 8 days were used for the thymidine phosphorylase and the nucleoside deoxyribosyltransferase assays.

Experimental procedures were essentially as described previously (13). All incubations were at 37° C in a shaking water bath for 30 min. The following modifications should be notified.

Phosphorolysis of thymidine. Enzyme activity was assayed at pH 7.0, and controls containing Tris/HCl buffer instead of phosphate were included. The change of absorbancy was determined at 300 nm in a Hilger-Gilford spectrophotometer (13, 14, 20).

Thymidine-adenine transfer in whole cells. Cells were harvested from overnight blood agar plates in 0.05 M Tris/HCl buffer at pH 7.2. After being washed once the cells were resuspended in the same buffer and adjusted to an absorbancy at 525

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TABLE 1 *Search for Thymidine Phosphorylase Nucleoside Deoxyribosyltransferase and Thymidine Kinase in Cell free Extracts from Neisseria sp Control with Extracts from* E. coli *(13)*

Species	Strain	Thymidine phosphorylase		Nucleoside deoxyribosyltransferase		Thymidine kinase	
		Protein mg/assay	Thymine formed µmoles	Protein mg/assay	Deoxyribose formed µmoles	Protein mg/assay	Nucleot[ide] forme[d] µmole[s] TMP+T[DP] +TT[P]
N gonorrhoeae	1 a	0.85	–	0.85	–	0.17	–
N sicca	CN	1 15	–	1 15	–	0.23	–
N mucosa	M 1	0.95	–	0.95	–	0.19	–
N perflava	ATCC 10555	0.90	–	0.90	–	0 18	–
N flava	ATCC 14221	0.75	–	0.75	–	0.15	–
N subflava	ATCC 19243	0.90	–	0.90	–	0.18	–
N lactamica	1379	0 65	–	0.65	–	0 13	–
N flavescens	ATCC 13120	1 10	–	1 10	–	0.22	–
N cinerea	165/61	0.45	–	0.45	–	0.09	–
N elongata	M 2	0.95	–	0 95	–	0.19	–
N catarrhalis	ATCC 8176	0.75	–	0.75	–	0 15	–
N ovis	199/55	0.95	–	0.95	–	0.19	–
N caviae	ATCC 14659	0.75	–	0.75	–	0.15	–
E. coli	K 12	1.50	3 35	0 65	0.17	0.26	1 8[illegible]

* The *E. coli* extract for the thymidine kinase assay had been kept frozen at −20° C for 28 days.

nm of 1 000 in a Spectronic 20 spectrophotometer One ml bacterial suspension corresponds to approximately 5×10^8 cells or about 0.6 mg protein. The assay was performed with 0.5 ml of the suspension. Blanks consisted of the assay mixture minus adenine (13)

Thymidine kinase Enzyme activity was assayed in a total volume of 55 µl by mixing 4 µmoles Tris/HCl buffer pH 8.0 0.5 µmole ATP 0.5 µmole $MgCl_2$ 0.5 µmole 3-phosphoglycerate 0.0047 µmole (53 µCi/µmole) ^{14}C-2-thymidine and 20 µl crude extract. The reaction was stopped by cooling. The protein was precipitated with 50 µl cold 96 per cent ethanol followed by 5 µl 0.1 M potassium-EDTA and removed by centrifugation. Appropriate controls with deproteinized extracts were included.

Chromatographic procedure Cellulose thin layers (Eastman Chromagram with fluorescent indicator) were ruled in channels 2 × 20 cm, and the samples, 10 µl portions of the supernatants, were applied between the pencilled lines 2 cm from the bottom of the plate. Standards of thymidine, TMP TDP and TTP (thymidine monophosphate, thymidine diphosphate and thymidine triphosphate) 3 µg of each, were run in parallel paths, because thymidine nucleotides and adenosine nucleotides which are also formed during the reaction, overlap more or less in the solvent used. Thymidine was separated from the nucleotides in the isobutyric acid solvent of Krebs & Hems (16) Chromatograms were developed in 3 hours (17 cm) thymidine TMP TDP and TTP had R_F values of 0.73 0.53 0 29 and 0.17 respectively Fluorescence quenching patterns on dried chromatograms were located with ultraviolet light and marked with pencil. For additional accurate location of the radioactive spots the chromatograms were exposed to X-ray film for 2–4 days (13) The strips were cut and counted as previously in the scintillation counter (10 13) The counting efficiency was about 54 per cent. The labelling of the thymidine nucleotides was confirmed by applying 10 µl of the supernatant on the cellulose thin layers together with standards of TMP TDP and TTP The chromatograms were run in two dimensions with the isobutyric acid solvent in the first direction and the *n*-butanol-acetone-acetic acid-conc. NH_3–H_2O solvent in the second (13) When run for 3 hours in each direction the system gave good separation of the thymidine nucleotides and the adenosine nucleotides. The radioactive spots were located by radioautography The spots on the radioautograms corresponded to the spots of the ultraviolet absorbing standards on the chromatograms both in position and shape.

Chemicals were those used previously (13)

RESULTS

Enzyme determinations. Significant thymidine phosphorylase, nucleoside deoxyribosyltrans-

ferase (thymidine-adenine) or thymidine kinase activities were not detected in crude extracts of the *Neisseria* species listed in Table 1 under the experimental conditions used. Crude extracts from an *Escherichia coli* K 12 strain were very active by the same techniques (13) and these extracts were used as positive controls throughout the experiments (Table 1) In *E. coli* the thymidine-adenine transfer is found to be more active in whole cells than in extracts (18) The transfer reaction was accordingly also searched for in whole cells of neisseriae, but no activity could be found.

DISCUSSION

These experiments indicate that the present genus *Neisseria* including also the rodshaped *N elongata* and the "false neisseriae" species *N catarrhalis N ovis* and *N caviae* lacks the enzymes thymidine phosphorylase, nucleoside deoxyribosyltransferase and thymidine kinase.

Thymidine phosphorylase is of widespread occurrence in microorganisms (12 21) Lactobacilli, however seem to be devoid of thymidine phosphorylase (24)

The transfer of deoxyribosyl groups between purines and pyrimidines, which can be demonstrated in cell-free extracts of various bacteria has been explained by coupling of two separate phosphorylases, specific for purine and pyrimidine deoxyribonucleosides respectively and both present in the extracts (12) In contrast, lactobacilli have acquired a specific enzyme *trans*-N-deoxyribosylase which catalyzes a phosphate independent transfer of deoxyribose moieties between a wide variety of purines and pyrimidines (2, 17) In purine nucleoside phosphorylase negative strains of *E. coli*, purines do not enhance cleavage of thymidine, indicating that the *trans*-N-deoxyribosylase activity which is demonstrated in purine deoxyribonucleoside phosphorylase positive strains, is a result of the concerted actions of purine deoxyribonucleoside phosphorylase and thymidine phosphorylase. This activity is partial ly destroyed by sonic treatment of the cells The phosphorolytic cleavage of thymidine is, on the contrary highly accelerated by this treatment (18)

Sonicated and frozen extracts from all the *Neisseria* species tested showed no phosphorolytic activity and no thymidine-adenine transfer activity could be found. Nor could any *trans*-N-deoxyribosylase activity in whole cells of the species be demonstrated.

Thymidine kinase also seems to be of wide spread occurrence in microorganisms. The mere existence of thymine or thymidine-requiring mutants shows that enzymes must exist for the conversion of thymine or thymidine to thymidine monophosphate (6, 8 15 19 22) No other route seems to exist in microorganisms for thymine or thymidine to TMP than by thymidine kinase This path way is a salvage mechanism not universally found in nature, however It is absent in *Neurospora Aspergillus Saccharomyces* and in the parasite *Plasmodium* (7 23) as well as in the *Neisseria* species studied.

In a recent investigation some Gram nega tive, saccharolytic, oxidase positive rod shaped bacteria without genetic affinities to *Neisseria* or to "classical" asaccharolytic species of *Moraxella* revealed high thymidine phosphorylase activity in sonicated and frozen extracts. The thymidine-adenine transfer ac tivity in the same extracts was low Whole cells, on the contrary catalyzed an efficient transfer of the deoxyribosyl group from thymidine to adenine. In the same organisms, ac tivities corresponding to thymidine kinase were easily detected. However bacterial spe cies unquestionably belonging to genus *Moraxella* on genetic grounds, were negative in these respects, except for observation of weak thymidine kinase activity in one strain (*S Jyssum & K Bøvre* to be published)

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SEARCH FOR THYMIDINE PHOSPHORYLASE, NUCLEOSIDE DEOXYRIBOSYLTRANSFERASE AND THYMIDINE KINASE IN *MORAXELLA*, *ACINETOBACTER* AND ALLIED BACTERIA

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Eighteen strains of Gram-negative, rodshaped bacteria, including *Moraxella kingii*, "New *Moraxella*" *Bijsterveld*, "New *Moraxella*" *Sutton et al.*, *M. nonliquefaciens*, *M. lacunata*, *M. bovis*, *M. osloensis*, *M. phenylpyruvica*, *M. atlantae* (new species to be described), *M. urethralis* (tentative designation) and *Acinetobacter*, were examined for enzyme activities corresponding to thymidine phosphorylase, nucleoside deoxyribosyltransferase and thymidine kinase. The two strains of *M. kingii* examined as well as "New *Moraxella*" *Bijsterveld* and "New *Moraxella*" *Sutton et al.* revealed activities corresponding to the three enzymes. One of two *M. nonliquefaciens* strains, the type strain, was found to contain a thymidine kinase of low activity, but had no activities corresponding to the enzymes thymidine phosphorylase or nucleoside deoxyribosyltransferase. Significant activities of any of the three enzymes were not detected in the other strains examined.

In previous reports it has been shown that several species of *Neisseria*, including also the group of "false neisseriae", *N. catarrhalis*, *N. ovis* and *N. caviae**, lack the enzymes known to be involved in the utilization of exogenous thymine or thymidine for incorporation into DNA (25, 26). These enzymes are thymidine phosphorylase EC 2.4.2.4, nucleoside deoxyribosyltransferase EC 2.4.2.6 and thymidine kinase EC 2.7.1.21 (1).

For genetic and taxonomic reasons it was of interest to find out if the lack of these enzymes is shared by species belonging to the genera *Moraxella* and *Acinetobacter*. A representative material of species and strains of these genera and organisms possibly related to them were selected for the present investigation.

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* These three species are only temporarily assigned to genus *Neisseria*; both *Moraxella* and *Branhamella* have been proposed as new genus designations for these organisms ( ).

MATERIALS AND METHODS

Organisms. Eighteen strains of *Moraxella*, *Acinetobacter* and allied bacteria were examined (Table 1): "New *Moraxella*" *Bijsterveld* (4), "New *Moraxella*" *Sutton et al.* (36), two strains each of *M. kingii* (16, 20), *M. nonliquefaciens* (9, 10), *M. lacunata* (19), *M. bovis* (9) and *M. osloensis* (10), one strain each of *M. phenylpyruvica* (11), *M.*

TABLE 1 *Organisms Examined Amount of Protein in the Extracts used for Assay and the Occurrence of Thymidine Phosphorylase Nucleoside Deoxyribosyltransferase and Thymidine Kinase Activities*

Species	Strains*	Colony type and fimbriation variants*	Protein mg/ml extract§	Enzyme activities†		
				Thymidine phosphorylase	Nucleoside deoxyribosyl transferase	Thymidine kinase
M kingii	4177/66 = ATCC 23330 = NCTC 10529 (type strain)	N-b	10.0	+	+	+
	A1702	N-b	9.0	+	+	+
"*New Moraxella*"	*Bijsterveld*		10.0	+	+	+
	Sutton et al.		12.0	+	+	+
M n nliquefaciens	4663/62 = ATCC 19975 = NCTC 10464 (type strain)	SC-a	1) 6.5	–	–	+
			) 17.5	–	–	+
	3067/66	N-a	9.3	–	–	–
M lacunata	ATCC 17967 (type strain)		6.5	–	–	–
	ATCC 17952 = NCTC 7911 (biotype *liquefaciens*)		12.5	–	–	–
M bovis	ATCC 10900 (type strain)	SC-a	6.5	–	–	–
	3	N-a	4.8	–	–	–
M osloensis	A1920 = ATCC 19976 = NCTC 10465 (type strain)		8.5	–	–	–
	5873		8.5	–	–	–
M phe ylpyruvica	2863 = ATCC 23333 = NCTC 10526 (type strain)		10 0	–	–	–
M tlant	5118		7 0	–	–	–
M urethralis	WM20		13.5	–	–	–
Acinetobacter	ATCC 17985		6.0	–	–	–
	ATCC 17988		5.5	–	–	–
	BD4		3 1	–	–	–

M. = *Moraxella*, "New *Moraxella*" see Discussion *M atlantae* new species to be described by *J E. Fuglesang* & *K. Bøvre* *M ur thralis* tentatively designated species (29) N = mainly nonfimbriated cells in nonspreading/noncorroding colony type SC = mainly fimbriated cells in spreading/corroding colony type a, b = particular clones or cell lines" see references in Materials and Methods for characteristics and origin of species, strains and variants

§ Protein was determined by the procedure of *Lowry et al.* (30)

† Experimental conditions described in the legends to T bles 2–6

lluntes (new species to be described by *J. E. F. g-lessng & K. Bovre*) and *M. urethralis* (tentative designation 29) and three strains of *Acinetobacter* (8 24). *Escherichia coli* K12 was used in some experiments for comparison (25 26).

Cell free extracts and whole cell preparations. Cells were harvested from the surface of blood agar plates after incubation for 20 hours at 33 C in a very humid atmosphere. The rest of the procedure was as described before (25 26).

Experimental procedures for enzyme determinations. The experimental methods were as described previously (25 26). d-CTP-di-Na was obtained from Koch-Light Labs. Ltd. Bucks. U.K. Other wise the chemicals were the same as those used before (25). Enzyme activity is expressed as nmoles of product formed per mg of protein at the time indicated in the tables. The thymidine kinase tivity is given as the sum of nucleotides formed, TMP + TDP + TTP (thymidine monophosphate + thymidine diphosphate + thymidine triphosphate).

RESULTS

Enzyme Determinations

Thymidine phosphorylase. Significant thymidine phosphorylase activity was detected in crude extracts from both strains of *M. kingii*, "New *Moraxella*" *Bijsterveld* and "New *Moraxella*" *Sutton et al.* as shown in Table 2. None of the other strains listed in Table 1 were found to contain activities corresponding to this enzyme in their extracts.

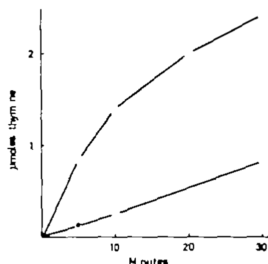


Fig. 1. Activities corresponding to thymidine phosphorylase in extracts from *M. kingii* A1702 (● — ●) and from "New *Moraxella*" *Bijsterveld* (○ — ○). Conditions of the experiments as in Table 2.

Fig 1 shows the gradual liberation of thymine from thymidine with time in the presence of extract from *M. kingii* A1702 and from "New *Moraxella*" *Bijsterveld*.

The activity of thymidine phosphorylase seems to differ considerably from one strain to another. In *M. kingii* A1702 the enzyme activity is taken to be linear for 5 minutes,

TABLE 2. *Thymidine Phosphorylase Activity in Cell-free Extracts from* Moraxella kingii "New Moraxella" Bijsterveld *and* "New Moraxella" Sutton et al.

Strain	Protein mg/assay	Time of incubation min	Thymine formed nmoles	Enzyme activity nmoles thymine formed per mg protein at the time indicated
M. kingii 4177/66	1.0	30	2670	2670
M. kingii A1702*	0.9	5	860	955
		30	2430	2700
"New *Moraxella*" *Bijsterveld*§	1.0	5	130	130
		30	830	830
"New *Moraxella*" *Sutton et al.*§	1.2	30	2280	1900

* The extracts stored at 20 C for 24 hours.

§ The extracts new. The enzyme activity was assayed in a total volume of 1.1 ml by mixing 160 μmoles of K-phosphate buffer pH 7.0, 2 mg thymidine and 0.1 ml crude extract. Controls containing Tris/HCl buffer instead of phosph te were sed as blanks. Incubation in a shaking water bath at 37 C. Samples of 0.. ml were mixed with 2.4 ml 0.3 M NaOH. Phosphorolysis of thymidine was determined from the change of absorbance t 300 nm (25 26 33).

whereas the activity in the extract from "New *Moraxella*" Bijsterveld shows linearity within 30 minutes under the experimental conditions used (Fig 1) The activity of thymidine phosphorylase in the extract from *M kingii* amounts to 955 at 5 minutes, and to 130 only in the extract from "New *Moraxella*" *Bijsterveld* (Table 2) The enzyme activities at 30 minutes are given for the strains listed in Table 2. The activity of thymidine phosphorylase from the *M kingii* strains 4177/66 and A1702 seems to be the same. The activity of this enzyme in "New *Moraxella*" *Sutton et al.* is about twice as high as that in "New *Moraxella*" *Bijsterveld*

Nucleotide deoxyribosyltransferase Attempts to demonstrate activities corresponding to this enzyme were performed with crude extracts or with whole cells when the crude extracts showed no activity or the activity was very low Of the strains listed in Table 1 only *M kingii* "New *Moraxella*" *Bijsterveld* and "New *Moraxella* *Sutton et al.* were found to have nucleoside deoxyribosyltransferase activity Deoxyribose is transferred at a linear rate from thymidine to adenine during 30 minutes, both in extracts and in whole cells (Fig 2) The activity of the enzyme seems to be nearly the same in extracts from the two strains of *M kingii*. Phosphorolytic as well as transfer activities in the extract from "New *Moraxella*" *Bijsterveld* are

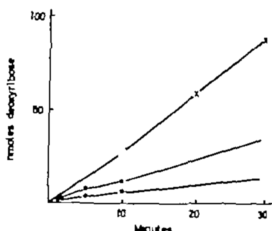


Fig 2 Activities corresponding to nucleoside deoxyribosyltransferase in extract (x — x) from *M kingii* A1702 and in extract (o — o) and whole cells (• — •) from "New *Moraxella*" *Bijsterveld*. Conditions of the experiments as in Tables 3 and 4.

only about one seventh of those found in *M kingii*, whereas these activities in the extract from "New *Moraxella*" *Sutton et al.* are about the double of those in the extract from "New *Moraxella*" *Bijsterveld* (Tables 2 and 3) The deoxyribonucleoside transfer activity in "New *Moraxella*" *Bijsterveld* and *Sutton et al.* was confirmed by using whole cells in the assay The activity of nucleoside deoxyribosyltransferase is several times higher in the intact cells of the organisms than in extracts (Tables 3 and 4) This is in accordance with the results from experiments with *E coli* (32)

TABLE 3 *Nucleoside Deoxyribosyltransferase Activity in Cell free Extracts from* Moraxella kingii, *New* Moraxella Bijsterveld *and "New* Moraxella" Sutton et al.

Strain	Protein mg/assay	Deoxyribose formed nmoles	Enzyme activity nmoles deoxyribose formed per mg protein in 30 min
M kingii 4177/66*	1 0	89.5	89 5
M kingii A1702*	0 9	88.8	98.6
"New *Moraxella*" *Bijsterveld*‡	1.0	13 1	13 1
"New *Moraxella*" *Sutton et al.*‡	1 2	35 0	29 1

* The extracts stored at −20 C for 24 hours,

‡ The extracts new The enzyme activity was assayed in a total volume of 2 5 ml by mixing 100 µmoles Tris/HCl buffer pH 7.2 2.5 mg thymidine 0.5 mg adenine and 0.1 ml crude extract. Controls without adenine were used as blanks. Incubation at 37 C in a shaking water bath. Aliquots of 0.65 ml were pipetted into 0.05 ml 70 per cent perchloric acid followed later by 0.1 ml of 0.5 M HClO Deoxyribose was determined according to *Burton* (7) with deoxyribose as a standard (25)

TABLE 4 *Nucleoside Deoxyribosyltransferase Activity in Intact Cells from "New Moraxella" Bøvre and "New Moraxella" Sutton et al.*

Strain	Protein mg/assay	Deoxyribose formed nmoles	Enzyme activity nmoles deoxyribose formed per mg protein in 30 min
"New Moraxella" Bøvre	0.3	35.6	118
"New Moraxella" Sutton et al.	0.4	121.2	303

A cell suspension of 0.5 ml in 0.05 M Tris/HCl buffer pH 7.2 was used in the assay. The suspension was adjusted to an absorbancy at 525 nm of about 1.000 in a Spectronic 20 spectrophotometer corresponding to approximately 5×10^{9} cells per ml (26). Otherwise the experimental system was as in Table 3 (25). Protein was determined by the procedure of *Lowry et al.* (30).

Thymidine kinase. The presence of activity corresponding to thymidine kinase was demonstrated in both strains of *M. kingii* "New *Moraxella*" *Bøvre*, "New *Moraxella*" *Sutton et al.* and in the *M. nonliquefaciens* strain 4663/62 (Table 5). None of the other strains listed in Table 1 were found to have significant activities corresponding to thymidine kinase in their crude extracts. The activity of the enzyme differs greatly from one strain to another. Under the experimental conditions shown in Table 5 the activity is taken to be linear during 5 minutes of incubation (Fig. 3, 4, 5). The activity is about 80 times higher in *M. kingii* A1702 than in *M. nonliquefaciens* 4663/62 and about 20 times

TABLE 5. *Thymidine Kinase Activity in Cell-free Extracts from* Moraxella kingii, *New* Moraxella Bøvre, *New* Moraxella Sutton et al. *and* Moraxella nonliquefaciens *Strain 4663/62*

Strain	Protein mg/assay	Time of incubation min	Nucleotides formed nmoles TMP + TDP + TTP	Enzyme activity nmoles nucleotides formed per mg protein at the time indicated
M. kingii 4177/66	0.10	30	3.52	35.20
M. kingii A1702*	0.09	5	1.38	15.33
		30	2.92	32.44
New Moraxella Bøvre§	0.20	5	0.15	0.75
		30	0.80	4.00
New *Moraxella Sutton et al.*§	0.24	30	0.73	3.04
M. nonliquefaciens 4663/62*	0.13	5	0.03	0.23
		30	0.11	0.84
M. nonliquefaciens 4663/62§	0.35	5	0.05	0.14
		30	0.25	0.71

* The extracts stored at −20 °C for 8 (*M. nonliquefaciens*) and 14 (*M. kingii*) days.
§ The extracts new. The enzyme activity was assayed in a total volume of 55 µl by mixing: 4 µmoles of Tris-HCl buffer pH 8.0, 0.5 µmole ATP, 0.5 µmole $MgCl_2$, 0.5 µmole 3-phosphoglycerate and 0.0047 µmole (53 µCi/µmole) ^{14}C-2-thymidine. The reaction was initiated by the addition of 10 or 20 µl crude extract. The incubation was at 37 °C in a shaking water bath. Protein was precipitated with 50 µl 96 per cent ethanol followed by 5 µl 0.1 M potassium-EDTA in an ice water bath. Thin-layer chromatography was performed with 10 µl of the supernatant in the isobutyric acid solvent of *Krebs & Hems* (28). Autoradiography of the chromatograms was used for accurate location of the radioactive spots. The chromatograms were [illegible] and counted in the scintillation counter. Appropriate blanks with deproteinized extract were run on the same thin layer plates (5, 26).

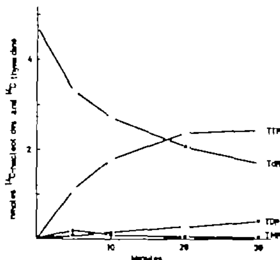
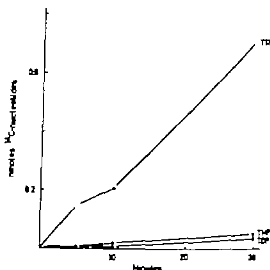


Fig 3 Thymidine kinase activity in extract from *M kingii A1702. The extract also showed activities* corresponding to TMP and TDP kinase. Disappearance of ^{14}C-2 thymidine and labelling of TMP TDP *and* TTP *The thymidine (TdR) is the sum* of thymidine not metabolized and thymine formed by the phosphorolytic cleavage of thymidine In the isobutyric acid solvent system (28) thymine overlaps with thymidine. Conditions of the experiment as in Table 5.



F g 4 Thymidine kinase activity in extract from "New *Moraxella*" *Bijsterveld* The extract also showed activities corresponding to TMP and TDP kinase. Conditions of the experiments as in Table 5

higher than that found in "New *Moraxella Bijsterveld* (Table 5) For comparison the activities after 30 minutes of incubation are given. The two strains of *M kingii*, 4177/66 and A1702 seem to have nearly the same activity and so do the two "New *Moraxella*" strains (Table 5)

In the experiments with crude extracts from the thymidine kinase positive strains there is a rapid accumulation of TTP (Figs. 3 4 5) There is a stepwise addition of orthophosphate to thymidine to yield successively TMP TDP and TTP The last two phosphorylations are functions of TMP kinase EC 2 7 4 4 and TDP kinase EC 2.7 4 6 (1)

Okazaki & Kornberg (33 34) demonstrated that Mn^{++} and certain nucleotides activated the *E. coli* thymidine kinase Among the nucleotides, d-CTP (deoxycytidine triphosphate) was *the most effective activator* The activity of thymidine kinase in extracts from *M nonliquefaciens* 4663/62 was low and no significant thymidine kinase activity could be demonstrated in strain 3067/66 under the experimental conditions used. Accordingly the activities were controlled with Mn^{++} or d-CTP added to the incubation mixture. Table 6 shows that Mn^{++} has a striking effect on the kinase activity in extracts from *M nonliquefaciens* 4663/62. The addition of Mn^{++} results in an increase of activity to 280 per cent of that with Mg^{++} only In *M nonliquefaciens*

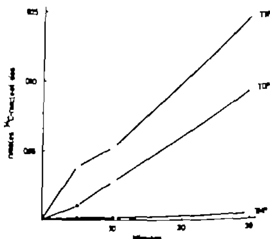


Fig 5 Thymidine kinase activity in extract from *M nonliquefaciens* 4663/62. The extract also showed activities corresponding to TMP and TDP kinase. Conditions of the experiment as in Table 5.

TABLE 6. *Thymidine Kinase Activity in Crude Extracts from Moraxella nonliquefaciens Strain 4663/62 and from E. coli K12 (26)*

Strain	Protein mg/assay	Chemical added	Nucleotides formed in 30 min TMP + TDP + TTP mmoles	Activity per cent
M. nonliquefaciens	0.35	none	0.157	100
		MnCl 2.1 mM	0.459	290
		d-CTP 5.0 "	0.033	21
		TTP 8.3	0.032	20
		TTP 0.83	0.176	112
		TTP 0.08	0.204	130
E. coli §	0.26	none	1.535	100
		d-CTP 5.0 mM	1.832	119
		TTP 8.3	0.008	0.5
		TTP 0.83	0.007	0.4
		TTP 0.08	0.801	52

The extract stored at −20° C for 1½ month and for 4 months §. The enzyme activity was assayed in a total volume of 60 µl. Conditions of the experiments as in Table 5.

3067/66 no activity was found even upon the addition of Mn^{++}.

The activating effect on the *E. coli* enzyme of d-CTP at a concentration of 5 mM is demonstrated (Table 6). Contrary to this, d-CTP has an inhibitory effect on the *M. nonliquefaciens* enzyme at the same concentration (Table 6).

Thymidine kinase from a variety of sources has been found to be subject to a strong feedback inhibition from TTP (5, 6, 13, 14, 22, 34). Table 6 shows that TTP has a strong inhibitory effect on the activity of the enzyme from *E. coli* in all the concentrations used. TTP also inhibits the enzyme from *M. nonliquefaciens* at a concentration of 8.3 mM. Contrary to the effect on the *E. coli* enzyme, TTP at concentrations of 0.83 and 0.08 mM stimulates the kinase activity in extracts from *M. nonliquefaciens* 4663/62.

DISCUSSION

In the present investigation thymidine phosphorylase was found to be lacking in most of the *Moraxella* strains as well as in *Acinetobacter*. The enzyme acti[illegible] was searched for in sonic cell extracts [illegible] since the phosphorolytic cleavage of th[illegible]idine is reported to be highly accelerated by sonic treatment of the cells (32). In the thymidine phosphorylase positive strains *M. kingii*, "*New Moraxella*" *Bøvre*, and "*New Moraxella*" *Sutton et al.* the phosphorolytic cleavage of thymidine was easily demonstrated with thymine formed in µmole amounts (Table 2).

The transfer of deoxyribosyl groups between purines and pyrimidines is either phosphorolytic or nonphosphorolytic. In *E. coli* (32) and other bacteria (23) the transfer reaction is explained by a coupling of two separate phosphorylases, specific for purine and pyrimidine deoxyribonucleosides respectively. In lactobacilli however the *nonparticipation* of phosphate has been established. The transfer is mediated by a specific *trans* N-deoxyribosylase in these bacteria (3, 31). No significant transfer of the deoxyribosyl group between thymidine and adenine was demonstrated in extracts or whole cells in most of the *Moraxella* strains and in the three strains of *Acinetobacter* listed in Table 1. Only *M. kingii* and the two "New *Moraxella*" strains showed activities corresponding to the purine-pyrimidine deoxyribosyltransferase. The activity in *M. kingii* cells was easily detected even in sonic extracts which had been stored at −20° C. No transfer activity was

found in a crude extract from "New *Moraxella* Bøvre" that had been kept frozen for 8 days, whereas unfrozen extracts and whole cells had pronounced activities. The activity was about 10 times higher in whole cells than in extracts (Tables 3 and 4). This is in agreement with the findings in *E. coli* (32). In the present investigation the transfer of the deoxyribosyl group was measured with adenine as acceptor since no other purine seems to act as a better acceptor in any of the transfer reactions from thymidine, phosphorolytic or nonphosphorolytic (3, 32). The transfer reaction found in *M. kingii* and "New *Moraxella*" seems to be of the phosphorolytic type, since thymidine phosphorylase activity is present in the extracts together with a transfer reaction of the deoxyribosyl group from thymidine to adenine.

Thymidine kinase is recognized as being a salvage enzyme found in a variety of microorganisms (13, 15, 27, 33), in an insect (6) and in mammalian systems (22, 37). Thymidine kinase is reported to be absent from microorganisms like fungi (17), some protozoa (17, 18) and bacteria (25, 26). Significant thymidine kinase was demonstrated in crude extracts from *M. kingii*, "New *Moraxella*" and *M. nonliquefaciens* 4663/62. None of the other *Moraxella* or *Acinetobacter* strains showed activities corresponding to this enzyme. The activity in extracts from *M. nonliquefaciens* 4663/62 was low when the assay system contained Mg^{++} as the only divalent cation. The addition of Mn^{++} enhanced the activity considerably and more than found in the *E. coli* system (33). The thymidine kinase in crude extracts from *Bacillus megaterium* has, on the contrary, no demonstrable divalent cation requirement (38). In most other systems Mg^{++} is reported to be the metal required almost exclusively for maximum activity (5, 6, 39). Strain 3067/66 of *M. nonliquefaciens* failed to show any thymidine kinase activity even with the addition of Mn^{++}. Whether this is due to a mutation in this strain or to unfavourable experimental conditions is not known. The possibility may also exist that phosphatases in the crude extracts break down the nucleotides at a faster rate than the nucleotides are formed.

The reported activation of thymidine kinase by d-CTP found in *E. coli* (34) and in *Lactobacillus acidophilus* (14) was not demonstrated with the *M. nonliquefaciens* enzyme in the concentration used. On the contrary, d-CTP had an inhibitory effect. Inhibition of thymidine kinase by d-CTP has also been reported from mammalian systems (22, 37) and from an insect (6).

An inhibition by TTP on the thymidine kinase activity seems to be a common characteristic of all systems (5, 6, 13, 14, 22, 34). With the thymidine kinase from *M. nonliquefaciens* the effect of TTP on the activity seems to be a matter of the concentration of the nucleotide. TTP in high concentration inhibited the enzyme. When the concentration was one tenth or one hundredth of the inhibitory one, a stimulating effect was achieved. The experimental conditions used in the assay of the thymidine kinase from *M. nonliquefaciens* are obviously far from the optimal ones. The enzyme will be the subject of further investigation in this laboratory. *M. nonliquefaciens* strain 4663/62 may serve as an excellent organism for specific radioactive labelling of DNA by thymidine since no thymidine phosphorylase could be demonstrated. Neither could any transfer of deoxyribose between thymidine and adenine be found. *Bean & Tomasz* (2) have demonstrated the lack of these enzymes in *Diplococcus pneumoniae* and obligate thymidine auxotrophs have also been isolated from *Pseudomonas acidovorans* (27).

The finding of activities corresponding to the three enzymes in *M. kingii* and the two strains labelled "New *Moraxella*" is of considerable taxonomic interest, since these organisms also in other ways differ from main characteristics of the genus *Moraxella* (21). Thus, they are outstanding by their production of acid from carbohydrates. The two strains "New *Moraxella*" Bøvre (4) and "New *Moraxella*" Sutton et al. (36) can on the basis of genetic transformation be considered as members of one and the same

species, separate from *M. kingii*. Like the latter species, "New *Moraxella*" lacks genetic affinities to other moraxellae and "false neisseriae" (*J. E. Fuglesang, S. D. Henriksen & K. Bøvre* to be published).

Our sincere thanks are due to Dr. *S. J. Eik* who made facilities available for the development of films at the X-ray section, Pediatric Clinic, Rikshospitalet, Oslo.

ADDENDUM

After submitting this paper for publication, we became aware of the very recent report by *S. Øverbø & K. Kleppe* (J. Bact. *116* 331–336 1973) on the pyrimidine metabolism in *Acinetobacter calcoaceticus*. The results seem to be in agreement with our findings.

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After submitting this paper for publication, we became aware of the very recent report by *S. Gurebe* & *A. Kleppe* (J. Bact. 116: 551–556, 1973) on the pyrimidine metabolism in *Acinetobacter calcoaceticus*. The results seem to be in agreement with our findings.

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MICROBIOLOGICAL ASSAY OF MIXTURES OF AMPICILLIN AND CLOXACILLIN IN PLASMA AFTER SEPARATION BY HIGH VOLTAGE ELECTROPHORESIS

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A method for the quantitative determination of plasma concentrations of ampicillin and cloxacillin in mixture is described. The antibiotics were separated by high voltage paper electrophoresis. The high affinity of cloxacillin for albumin seriously interfered with the electrophoretic separation of the antibiotics and the quantitation of cloxacillin. Therefore, prior to electrophoresis, the plasma proteins were digested with trypsin to release cloxacillin. Subsequent to the electrophoretic separation the antibiotics were developed microbiologically by agar diffusion using the separated spots on the paper as diffusion centres. The inhibition zones were clearly visible after staining and could be quantitated. The method was evaluated statistically and showed good agreement with the paper disc diffusion method.

Assays of small quantities of mixtures of antibiotics in body fluids have been reported by several investigators. One method is to use bacterial strains selected to be sensitive only to one of the drugs (9 11 13) Another method used is that of microbiological determination, subsequent to chemical inactivation of all drugs but one (6 9 12) Alternatively the drugs can be assayed following separation of the antibiotics by chromatography (5 10) or electrophoresis (8) In our hospital, neonatal septicaemia is initially treated with streptomycin ampicillin and cloxacillin in combination. The concentrations of streptomycin and cloxacillin have been determined by the disc diffusion method developed by *Jalling et al.* (6) The quantitation of ampicillin by using selected bacterial strains failed, however as the presence of cloxacillin potentiated the antibacterial effect of ampicillin (6) *Naumann et al.* (11) reported the successful results obtained in experiments in which a selected bacterial strain had been used for determination of ampicillin in the presence of oxacillin. In the present study high voltage electrophoresis was applied for the separation of ampicillin and cloxacillin. The high affinity of cloxacillin for albumin, however results in inhibition zones seriously disturbed in the subsequent microbiological assay This problem was overcome by digestion of the plasma proteins by trypsin prior to the electrophoretic separation The results

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were compared to those obtained with a disc diffusion assay

MATERIALS AND METHODS

I *Microbiological Assay of Ampicillin and Cloxacillin Subsequent to Separation by High Voltage Electrophoresis (the Electrophoretic Method)*

Antibiotics The sodium salts of ampicillin (Doctacillin, Astra, Sweden) and cloxacillin (Ekvacillin, Astra, Sweden) were dissolved in phosphate buffer pH 7 4–7.5 according to *Dulbecco & Vogt* (5) and in pooled human plasma from 4–6 healthy donors, pH 7 4–7.5 If needed, the pH of the plasma was adjusted with 0.1 M potassium dihydrogenphosphate. From these stock solutions, (ampicillin 100 μg/ml, cloxacillin 400 μg/ml) standard solutions were prepared. All solutions were stored at –70 C.

Protein digestion. Trypsin (Sigma T 1583) was added to the plasma standard solutions (0.5 ml) to give a final concentration of 2 per cent. These solutions were incubated for 6 hours at +37 C. The digestion was interrupted by freezing the samples at –70 C

High voltage electrophoresis. The electrophoresis equipment was made by AB Analysteknik, Vallentuna Sweden. A piece of paper Whatman No 1 (33 × 47 cm) was soaked in 0 075 M Tris-maleate-buffer pH 5 6. Excess buffer was removed by squeezing the paper between filterpapers. The Whatman paper was then placed in the electrophoresis apparatus and 10 μl of samples were applied on points with a micropipette (Beckman Co.) The electrode vessels were filled with 0.075 M Tris-maleate-buffer pH 5.6 and the electrophoresis was continued for 90 min with a voltage gradient of 50 V/cm.

Microbiological development of the separated components 350 ml of agar (Bacto Penassay Seed Agar pH 7.5 Difco) containing approximately 7 × 10⁸ bacteria of the indicator strain (*Staphylococcus albus KS 462*) per ml agar was poured on a glass plate (50 × 35 × 0.3 cm) The plate was placed in a closed tray and stored at +4 C until use (not longer than 5 days)

After electrophoresis the paper was applied on top of the agar plate and left at room temperature for 30 min to allow prediffusion and subsequently incubated at +37 C for 18 hours. The paper was removed and the zones of bacterial growth inhibition were visible In order to precipitate the proteins the glass plate was soaked for 10 min in a mixture of 1000 ml of saturated solution of picric acid in water and 110 ml of glacial acetic acid. Subsequently the plate was washed in 99.5 per cent ethanol. A wet filter paper (Munktell No 20) was placed on top of the agar layer care being taken that no air bubbles were trapped. Drying at +70° C resulted in a thin agar film which was stained for 10 min in a 0.2 per cent solution of Coomassie Brilliant Blue R (Gurr London) in methanol/glacial acetic acid/water (5 1:5) The plate was then rinsed in the same solvent and dried The diameters of the clear inhibition zones in the blue background were measured with a sliding caliper

Based on the measurements of samples with known concentrations, standard curves were plotted on semilogarithmic paper The curves were used for the quantitation of the concentration of unknown samples containing ampicillin and cloxacillin according to the diameters of their inhibition zones. Diameters below 9 mm could not be measured satisfactorily due to the indeterminate edges.

II *The Disc Diffusion Method*

For comparison, some of the standard solutions of each penicillin were quantitated by the disc diffusion method of *Jalling et al.* (6)

The test medium (Bacto Penassay Seed Agar pH 6.6 Difco) was poured into plastic Petri dishes (14 cm in diameter) to an approximate depth of 4 mm. Test strain, inoculum and antibiotic solutions were the same as those described in section I Ten μl of these standard solutions were applied to paper discs, 5.5 mm in diameter The discs were then placed on the agar surface. After prediffusion for 30 min the plates were incubated overnight. The inhibition zones were measured and a standard curve could be constructed in a semilogarithmic diagram. Diameters below 7 mm were not measured due to low precision.

III. *Clinical Application*

Venous blood from two adults one receiving ampicillin 0 5 g i v and the other receiving cloxacillin 3 g i.v was collected in heparinized tubes

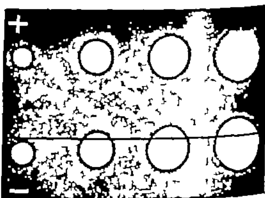


Fig 1 Inhibition zones of ampicillin (below application line) and cloxacillin (above application line) after separation by high voltage electrophoresis. Diluent phosphate buffer + anode – cathode.

Capillary blood from a 7 days old child receiving streptomycin 6.5 mg i.m., ampicillin 57 mg i.v. and cloxacillin 29 mg i.v. per kg body weight, was collected in heparinized capillary tubes. Blood samples were taken 1, 3 and 5 hours after drug administration and the plasma was collected after centrifugation. The plasma concentrations were determined by the electrophoretic method as described in section I. The samples containing only a single penicillin were also assayed by the disc diffusion method as described in section II, but the samples were not digested. The test strain used was *Staphylococcus aureus Oxford* st. aur. 209.

RESULTS

Assay by Microbiological Technique after High Voltage Electrophoresis

Inhibition zones of ampicillin and cloxacillin in mixture in different diluents (phosphate buffer, human plasma and human plasma treated with trypsin) are illustrated in Figs. 1–3. Cloxacillin moved towards the anode and ampicillin more slowly towards the cathode. The dark stained areas represent the plasma proteins, where albumin is closest to the anode. Streptomycin could not be visualized due to the unsuitable pH of the medium (4).

Fig. 1 illustrates the results obtained if the penicillins were dissolved in phosphate buffer. The inhibition zones for both penicillins were circular. If, however, the antibiotics were

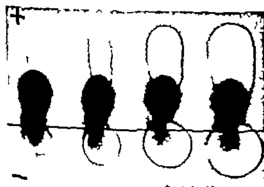


Fig. 2. Inhibition zones of ampicillin (below application line) and cloxacillin (above application line) after separation by high voltage electrophoresis. Diluent: human plasma. The dark areas represent the plasma proteins after staining with Coomassie Brilliant Blue. + anode, – cathode.

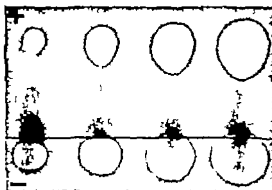


Fig. 3. Inhibition zones of ampicillin (below application line) and cloxacillin (above application line) after separation by high voltage electrophoresis. Diluent: human plasma digested with trypsin. The dark areas represent the partly digested plasma proteins after staining with Coomassie Brilliant Blue. + anode, – cathode.

dissolved in human plasma the inhibition zones for cloxacillin were not circular as a consequence of its binding to the plasma proteins as illustrated in Fig. 2. The inhibition zones for ampicillin, however, were circular in the presence of plasma proteins.

More circular inhibition zones were obtained when the plasma standard solution of cloxacillin was treated with trypsin (Fig. 3). The staining properties changed due to partial digestion of the proteins. The treatment with trypsin did not change the configuration of the inhibition zones of ampicillin.

Comparison of the Electrophoretic Method and the Disc Diffusion Method

The electrophoretic method was applied to standard solutions of each penicillin. In addition, each standard solution was analysed by the disc diffusion method. Fig. 4 illustrates the standard curves obtained by both methods and applying to ampicillin dissolved in phosphate buffer, human plasma, and human plasma treated with trypsin. It applies to each method that the curves were linear and almost superimposable.

Fig. 5 shows standard curves for cloxacillin obtained in the same way. Using the disc diffusion method, the inhibition zones in plasma would be smaller than those obtained in phos-

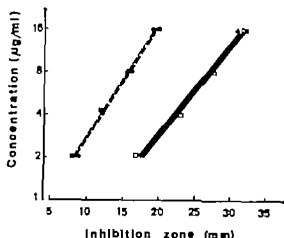


Fig 4 Standard curves determined by the electrophoretic method for ampicillin dissolved in phosphate buffer (○—○) human plasma (△—△) human plasma treated with trypsin (□—□) and determined by the disc diffusion method (filled symbols and dotted lines) Test strain *Staph. albus KS 462*

phate buffer After digestion, the antibacterial activity was found to be the same as that in phosphate buffer If compared with standard curves obtained in phosphate buffer and in digested plasma a parallel displacement of the standard curve in plasma would be observed. Using the electrophoretic method

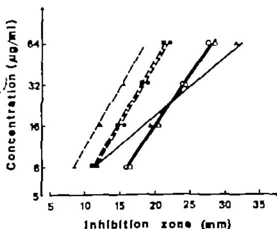


Fig 5 Standard curves determined by the electrophoretic method for cloxacillin dissolved in phosphate buffer (○—○) human plasma (△—△) human plasma treated with trypsin (□—□) and determined by the disc diffusion method (filled symbols and dotted lines) Test strain *Staph albus KS 462*

however the inhibition zones for cloxacillin in plasma would be pear shaped. Therefore they were approximately quantitated by extracting the root from the product of the lengths of the short and the long axis. By this method the standard curve in plasma is displaced and not parallel with those in phosphate buffer and in digested plasma.

As seen in Figs. 4 and 5 trypsin alone has no influence on the activity of ampicillin and cloxacillin or on the test strain.

Using the electrophoretic method, the measurable range of the concentrations of ampicillin was 2 to 125 µg/ml and of cloxacillin 8 to 200 µg/ml. The limit of the minimal measurable concentration of cloxacillin could sometimes be 6 µg/ml if the agar depth of the plate was very thin. The corresponding figures obtained by the disc diffusion method were 0.5 to 250 µg/ml and 2 to 500 µg/ml. Between these values, the standard curves applying to both antibiotics were still linear in the diagram.

With a view to evaluating the electrophoretic method, a statistical analysis was made and compared with the analysis of the disc diffusion method. For both antibiotics, ten identical series of each preparation of the standard solutions were analyzed by the electrophoretic method. All series of the same preparation were placed on a separate paper In addition, ten identical series of standard discs were prepared from the various preparations of both antibiotics. Each series was placed on a separate Petri dish. Least squares regression analysis gave correlation coefficients between 0.937 and 0.999 The 95 per cent confidence interval of the estimated concentrations of ampicillin and cloxacillin at the approximate midpoint of the concentration range was calculated (Tables 1 and 2) Using two standard series instead of one the 95 per cent confidence interval diminished in both methods. Three series usually gave little further improvement.

Clinical Application

Some samples from patients were compared in order to confirm the agreement between

TABLE 1 *The 95 per cent Confidence Interval of the Estimated Concentration of Ampicillin at the approximate Midpoint of the Concentration Range*

	The electrophoretic method								
	Phosphate buffer			Plasma			Trypsinated plasma		
	A	B	C	A	B	C	A	B	C
Number of standard series	1	2	3	1	2	3	1	2	3
Number of observations	4	8	12	4	8	12	4	8	12
Standard error of estimate	0.008	0 020	0.029	0 053	0 025	0.039	0 014	0 033	0.021
95 per cent confidence interval in per cent	+18 −21	+13 −15	+10 −1	+39 −65	+13 −15	+12 −14	+24 −31	+15 −17	+10 −10
	The disc diffusion method								
	Phosphate buffer			Plasma			Trypsinated plasma		
	A	B	C	A	B	C	A	B	C
Number of standard series	1	2	3	1		3	1	2	3
Number of observations	4	8	12	4	8	12	4	8	1
Standard error of estimate	0 002	0 008	0.007	0 005	0.012	0 011	0.023	0.010	0.004
95 per cent confidence interval in per cent	+9 −10	+8 −8	+6 −6	+15 −18	+9 −10	+7 −7	+28 −39	+8 −9	+4 −4

A One out of ten standard series chosen at random
B Two out of ten standard series chosen t random
C Three out of ten standard series chosen at random

the two methods. The results obtained in the two patients who received single penicillin administration are shown in Table 3 The plasma concentrations of ampicillin obtained by the electrophoretic method and by the disc diffusion technique are in good agreement. The concentrations of cloxacillin obtained by the two methods also agree, provided that the plasma had been digested prior to determination by the electrophoretic method.

Figs. 6 and 7 show the plasma concentrations in the child receiving the combined therapy of streptomycin ampicillin and cloxacillin. The plasma levels of ampicillin and cloxacillin were determined by the microbiological assay after separation by high voltage electrophoresis. Three determinations were made in the phase of elimination, as suggested by *Bordus* & *Jalling* (2) For clinical purposes, the plasma half lives were calculated from these three points by least squares regression analysis and were for ampicillin 3.2 hours and for cloxacillin 2.7 hours. The concentrations and half-lives obtained were of the same order as those observed by *Bordus* & *Jalling* in the newborn period (personal communication 1) The disc diffusion procedure did not allow quantitation of the two penicillins if administrated in combination.

DISCUSSION

In a previous report, the importance of control of antibiotic treatment with a view to ensuring therapeutic concentrations and avoiding toxic side effects was pointed out (6) By the paper disc micromethod it was possible to determine the concentration of streptomycin and cloxacillin individually but not that of ampicillin if all three drugs were administered simultaneously Thus we have

TABLE 2 *The 95 per cent Confidence Interval of the Estimated Concentration of Cloxacillin at the approximate Midpoint of the Concentration Range*

	The electrophoretic method					
	Phosphate buffer			Trypsinated plasma		
	A	B	C	A	B	C
Number of standard series	1	2	3	1	2	3
Number of observations	4	8	12	4	8	12
Standard error of estimate	0.001	0 006	0 028	0.002	0.008	0.007
95 per cent confidence interval in per cent	+4 −4	+8 −8	+11 −12	+10 −11	+7 −9	+5 −5

	The disc diffusion method								
	Phosphate buffer			Trypsinated plasma			Plasma		
	A	B	C	A	B	C	A	B	C
Number of standard series	1	2	3	1	2	3	1	2	3
Number of observations	4	8	12	4	8	12	4	8	12
Standard error of estimate	0 044	0 031	0.038	0.044	0.007	0.034	0.047	0.019	0 016
95 per cent confidence interval in per cent	+37 −59	+15 −17	+11 −19	+37 −59	+8 −8	+12 −14	+43 −77	+12 −13	+8 −9

A. One out of ten standard series chosen at random
B. Two out of ten standard series chosen at random
C. Three out of ten standard series chosen at random

TABLE 3 *Concentrations of Ampicillin and Cloxacillin in Two Patients as Determined by the Disc Diffusion Method and the Electrophoretic Method*

Antibiotic	Time after dose (hrs)	Plasma concentrations: Disc diffusion method (µg/ml)	Plasma concentrations: Electrophoretic method (µg/ml)
Ampicillin 0 5 g i.v	1	7 0	7 2
(Patients no 1)	3	<0.5	<2 0
	5	<0.5	<2 0
Cloxacillin 3 g i.v	1	50.0	47 5
(Patient no. 2)	3	6.2	6 0
	5	<2.0	<6 0

investigated the possibility of separating ampicillin and cloxacillin prior to microbiological assay *Lightbown & de Rossi* (8) have succeeded in separating several antibiotics by high voltage electrophoresis in agar These authors, however used a rather complicated apparatus. By means of a commercial high voltage electrophoresis *equipment* and using paper as the electrophoretic medium we have succeeded in assaying cloxacillin and ampicillin in plasma in one single operation.

However the high protein affinity of cloxacillin (7) seriously disturbed the inhibition zones in the following microbiological assay

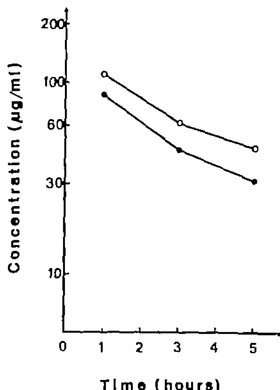


Fig 7 Plasma concentrations of ampicillin (o——o) and cloxacillin (●——●) after i.v. injection in a 7 days old girl. Doses ampicillin 37 mg i.v. and cloxacillin 29 mg i.v. per kg b.w. The determination was performed microbiologically after separation by high voltage electrophoresis.

special equipment. Digestion prolongs the procedure for 6 hours. Even so it is possible to deliver the result within 24 hours. The electrophoretic separation requires only 10 µl of plasma sample. The digestion, however increases the need of plasma to 500 µl. The electrophoretic method is less sensitive than the disc diffusion method probably because of a larger diffusion centre in the electrophoretic procedure. However the sensitivity can be increased by multiple applications of the sample. The disadvantages must be weighed against the advantage of the possibility of assaying related antibiotics in mixture. Furthermore, upon combined drug therapy the electrophoretic method including microbiological development can be used for the determination and identification of each antibiotic in mixture in one single operation.

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CULTIVATION OF A BACTERIAL FLORA ABLE TO PREVENT THE COLONIZATION OF *SALMONELLA INFANTIS* IN THE INTESTINES OF BROILER CHICKENS, AND ITS USE

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Mixed bacterial cultures from the ingesta of cocks grown under different circumstances were tested for their inhibitory effect on the colonization of *Salmonella infantis*. The cultures grown in one or more of three different media were given to chickens, which were inoculated with *S. infantis* one day or one week later. It was shown that the original media could be considerably simplified without causing any change in the preventive effect of the culture. An anaerobically incubated VL-medium was shown to be convenient for the cultivation of a flora inhibiting the colonization of *S. infantis* in the intestines of broiler chickens equally well at the age of one day and at that of one week. Meat broth or aerobically incubated VL-medium were shown to be unsuitable. The culture grown in a VL-medium was shown to be effective against *S. infantis* also when administered to the chickens with the drinking water.

It has previously been shown that the colonization of *Salmonella infantis* in the intestines of broiler chickens is effectively prevented by means of the intestinal contents of adult cocks (*Nurmi & Rantala* 1973) or of a mixed flora cultured from the same origin (*Rantala & Nurmi* 1973). The culture methods used, however, were complicated and involved a number of different broths. The purpose of the present work was to test various simplified media and methods of cultivating the mixed flora for the salmonella prevention in practice.

At the same time, the effect which the method used exerts on the colonization of *Salmonella infantis* in the intestines of chickens at the age of two days or of one week was also tested in order to test the efficiency of the method in several independent experiments.

For practical purposes, the possibility of applying the same treatment in drinking water was also studied.

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MATERIAL AND METHODS

The chickens, cocks, *Salmonella infantis* inocula and the methods used for the detection and identification of salmonellae in the samples were the same as in our previous work (*Nurmi & Rantala* 1973).

The mixed bacterial cultures for each experiment were prepared as follows. The media employed in experiment I were RCM (Oxoid), MRS- (*Man et al.* 1960) and VL-broths (*Barnes & Impey* 1971). Into all these media, inoculum was transferred from the small intestine and the caecum of a cock. The MRS- and RCM-media were in-

TABLE 1 *Numbers of* Salmonella infantis *in the Caecal Contents of One-week-old Chickens either Pretreated or not Pretreated with Cultured Flora from the Intestines of Cocks*

Experiment		I		II		III		IV		V		VI			
Groups		§ A	B	§ A	B	§ A	B	§ A	B	† A	B	† A	§ A_2	§ A_1	B
Culture conditions	MRS ROM VL	+ extracts anaer	–	VL + extracts anaer	–	VL anaer	–	VL anaer	–	VL anaer	–	VL anaer	VL anaer	meat broth	–
Number of salmonellas in 1 g of sample															
10^9									4		2				
10^8			2		9		2		13		8				2
10^7			9				7			1					2
10^6					1		1							1	1
10^5										1					
10^4						1		1		1				4	
By enrichment only				1									1		
Not detected		10		10		9		9		7		5	4		
Total number of chickens		10	11	11	10	10	10	10	17*	10	10	5	5	5	5
Statistical result	$\chi^2 =$	20.7		17.6		16.4		23.2		10.8			A_1+A_2 A_3 11.3		A_1+A_2 B 14.6
	P <	0.001		0.001		0.001		0.001		0.01			0.001		0.001 A_3 B no diff; t = 5.3 P < 0.001

A = pretreated at day 1 of age, challenged with 10^3 *S infantis* organisms at day 2
B = not pretreated, at day 1 of age, challenged with 10^3 *S infantis* organisms at day
† = pretreatment given with the drinking water
§ = pretreatment given by tube
* 3 chickens died during the experiment and were excluded from the data

oculated with the contents of the cock's crop. All media were supplemented with 5 per cent liver extract (w/v) 10 per cent fecal extract (w/v) (*Barnes & Impey* 1971) and 0.001 per cent hemin (w/v) (Fluka AG Buchs SG Switzerland) The cultures were mixed before feeding.

In experiments II and VII only the VL-medium supplemented with 5 per cent liver extract (w/v) and with 10 per cent fecal extract was used, and the inoculum was taken from the intestines only The medium for the cultivation of the flora in experiments III VI and VIII XIV was a VL-medium without additives, except in the group A_2 in experiment VI where the medium used was meat broth. Inoculum was taken in those experiments from the intestines only

The cultures were in each case transferred after 1–2 days growth to a new medium of the same kind, altogether 1–3 times. The incubation took place anaerobically except in experiment XIV where the culture was aerobically grown.

Each experiment comprised two groups: group A, which received the pretreatment, and group B, the control, which was treated in exactly the same way as group A except for the pretreatment. The chickens were pretreated with the cultures at the age of 1 day

The inoculations were usually made by tube into the crop. The approximate dose per chicken was 1 ml of the broth or mixture of the broths. In experiments V VIII and XIII and in group A_1 of experiments VI and XIV the cultures were administered with the drinking water 0.[illegible]–1 ml of the culture in 0 8-4 ml of water per chicken, left with the chickens over the night.

All chickens in experiments I–VI were inoculated with *Salmonella infantis* on the following day The approximate dose was 10^4 organisms to each chicken given by tube These chickens were sacrificed at the age of 7–10 days.

The chickens in experiments VII and IX–XIV were inoculated with *Salmonella infantis* at the age of 8 days, the dose being 10^6 salmonellae to each chicken, which is higher than the dose given to the chickens at the age of two days because the resistance of chickens increases rapidly with age. The chickens in experiment VIII were inoculated with the same dose (10^6) but at the age of two days All chickens in experiments VII–XIV were sacrificed at the age of about two weeks.

The caeca of all chickens were studied for salmonellas both quantitatively by dilution technique and by enrichment (*Nurmi & Rantala* 1973)

RESULTS

In experiment I the intestinal flora for the pretreatment was cultured in 3 different media, while in experiments II–IV only the VL-medium with (exp II) or without (exp III and IV) fecal and liver extracts was used. As seen in Table 1 they all gave the same protection and highly significant differences between the pretreated and non-pretreated groups. The detection of salmonellas in the pretreated groups was only occasional and the numbers were then comparatively small. All the chickens without pretreatment on these four groups had more than 10^6 salmonellae in 1 g of caecal contents.

In experiment V pretreatment given in drinking water the difference between the positive samples of the pretreated and non pretreated groups was highly significant (Table 1)

In experiment VI (Table 1) the difference between the number of positive samples (1/10) in groups A and A_1, pretreated with bacteria grown in a VL-medium, and the number of positive samples (5/5) in group A treated with bacteria grown in meat broth, was highly significant. The difference between the control group B and groups A + A was also highly significant. There is no difference between the numbers of positive samples in groups A and B (5/5 in each case) although the quantitative difference is highly significant so that even the meat broth grown culture in A appears to give some protection. There is no difference between the groups pretreated with the same culture by tube (A_1) or in the drinking water (A_2)

In experiments VII and IX–XIII (Table 2) there was a delay of 1 week between the pretreatment and the salmonella challenge. In experiment VIII the salmonella inoculum was 10^6 at the age of two days. The differences in the numbers of salmonella-positive samples and in the amounts of salmonellas in the samples between the pretreated and non-pretreated chickens were again significant or highly significant except in experiment XI where even in the control group 50 per cent of the samples showed no salmonellas.

All the samples of chickens pretreated with bacteria grown in VL-medium and of those not pretreated are compiled in Tables 3 and

TABLE 2. *Numbers of* Salmonella infantis *in the Caecal Contents of Two Weeks Old Chickens either Pretreated or not Pretreated with Cultured Flora from the Intestines of Cocks*

Experiment	VII		VIII		IX		X		XI		XII		XIII		XIV		
Group	‡ A	B	† A	B	‡ A	B	‡ A	B	‡ A	B	‡ A	B	‡ A	B	† A_1	‡ A_1	B
Culture condition	VL + extracts anaer	–	VL anaer	–	VL anaer	–	VL anaer	–	VL anaer	–	VL anaer	–	VL anaer	–	VL aer	VL aer	–
Number of salmonellas in 1 g of sample																	
10^9																	
10^8		1		9				2				5					1
10^7		1		1		3		5				6		4	1	1	4
10^6		1				3		2		1					2		1
10^5		3	1													2	
10^4			1			2	1			1					1	1	
By enrichment only		2	1		1			1	1	3				2	[illegible]	2	1
Not detected	10	2	6		8	2	9		9	5	9		10	3	1	1	
Total number of chickens	10	10	9*	10	9	10	10	10	10	10	9	11	10	9*	7	7	7
Statistical results																	
χ^2 =	13.4		9.8		9		16.4		3.8		20.4		10			A + A_1,B χ^2 = 1.1	
P <	0.001		0.01		0.01		0.001		0.05		0.001		0.01			P < 0.1 t = 2.7 P < 0.01	

A = pretreated at day 1 of age, challenged with 10^6 *S. infantis* organisms at day 8 in the others and at day 2 in group VIII

B = not pretreated at day 1 of age challenged with 10^6 *S.i f tis* organisms t day 8 in the others and at day 2 in group VIII

† = pretreatment given with the drinking water

‡ = pretreatment given by tube

* One chicken died during the experiment nd was excluded from the data

TABLE 3 *Numbers of* Salmonella infantis *in the Samples from the Caeca of Chickens Pretreated with Bacteria Grown in VL-medium and of Non pretreated Chickens Sacrificed at the Age of About 1 Week (Pooled Results of Experiments III–VI in Table 1)*

Number of *S infantis* in 1 g of sample	Numbers of chickens with the indicated counts of *S infantis* in samples from the caeca	
	pretreated	not pretreated
10^9	0	6
10^8	0	25
10^7	1	9
10^6	0	2
10^5	1	0
10^4	3	0
By enrichment only	1	0
Number of negative samples	34	0
Number of positive samples	6	42
Total number of chickens	40	42
	$\chi^2 = 60.8$	$P < 0.001$
	$t = 21.7$	$P < 0.001$

4 The difference between pretreated and non-pretreated chickens was highly significant in both 1 week and 2 weeks old chickens, both in the quantitative salmonella counts and in numbers of positive samples

In experiment XIV chickens were pretreated with bacteria grown aerobically in VL-medium. There was no significant difference in the numbers of salmonella-positive samples compared to the chickens without pretreatment. There was perhaps a suggestive reduction in the salmonella counts in groups A and A_4 compared with the control B

DISCUSSION

The method earlier used to prevent the colonisation of *Salmonella infantis* in the intestines of chickens by means of a flora cultured from the intestines of adult cocks was highly effective, but the method of cultivating the mixed flora was quite complicated. The bacteria were cultured in different media both anaerobically and aerobically the media were supplemented with different materials and the flora was taken from several parts of the intestine (*Rantala* & *Nurmi* 1973) For practical use, the methods were gradually simplified in the experiments described here. The results show that VL-medium without any additives, grown anaerobically is convenient for the cultivation of a mixed bacterial flora capable of preventing the colonisation of *Salmonella infantis* in the intestines of chickens. Neither the VL-medium grown aerobically nor meat broth grown anaerobically were suitable to the purpose. Although even these cultures gave some protection seen in the decrease in the numbers of salmonellas in the caeca of the chickens, there was no reduction in the numbers of salmonella-positive samples as compared with the control groups.

There were smaller differences in the num-

TABLE 4 *Number of* Salmonella infantis *in the Caeca of Chickens Pretreated with Bacteria Grown in VL-medium and in the Caeca of Chickens without Pretreatment Sacrificed at the Age of about 2 Weeks) (Pooled Results of Experiments VIII–XIII in Table 2)*

Number of *S infantis* in 1 g of sample	Numbers of chickens with the indicated counts of *S infantis* in samples from the caeca	
	pretreated	not pretreated
10^9	0	0
10^8	0	16
10^7	0	19
10^6	0	6
10^5	1	0
10	2	3
By enrichment only	3	6
Number of negative samples	51	10
Number of positive samples	6	50
Total number of chickens	57	60
	$\chi^2 = 62.2$	$P < 0.001$
	$t = 13.0$	$P < 0.001$

ber of salmonellas between the pretreated and control groups of the older chickens as compared to the experiments performed with younger chickens. (Compare Tables 2 and 4 with Tables 1 and 3) The smaller difference was due to some salmonella negative samples found in the control group as well. The resistance of the chickens has been shown to increase with the age (*Sadler et al* 1969)

An important practical point is that the cultured flora proved to be fully effective when given with the drinking water.

Strictly anaerobic organisms are known to constitute the dominant part of the caecal flora of adult birds (*Ochi et al.* 1964). Since both anaerobic conditions and a VL-medium are needed for protective cultures it seems that a high oxydation reduction potential or the presence of oxygen may be a limiting factor for the growth of bacteria able to prevent the colonisation of *Salmonella infantis* in the caecum of chicken.

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YEAST FUNGUS FLORA IN PATIENTS IN A GERIATRIC HOSPITAL

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In a 220-bed geriatric hospital in Helsinki, 427 specimens were taken for fungal culture from 155 aged persons in 1970–1973 Of this sample material, 44.0 per cent were positive for yeast growth. A total of 212 yeast strains, belonging to 11 species, were isolated. The strains were most generally isolated from sputum (66.7 per cent of specimens were positive for yeast) The incidence of occurrence of the most common yeast, *Candida albicans* was 26.5 per cent in the total specimen material (= 52.8 per cent of all isolated yeasts) The second in frequency was *Torulopsis glabrata* (21.2 per cent of isolated yeasts)

Yeast mycoses, especially those caused by species of *Candida* (candidoses), are mainly endogenous opportunistic infections. Risk groups in which predisposition to candidosis is high are known to be those including patients who usually are debilitated by a primary disease, particularly malignant neoplasms or leukaemia (*Bodey* 1966 *Kozinn et al.* 1969) Other conditions generally known to increase the morbidity in candidosis are, for example, diabetes, prolonged antibiotic and corticoid therapy and pregnancy (*Seelig* 1966 *Winner* 1966 *Utz* 1970) If old age be added to some of these factors a high incidence of yeasts and yeast infections may be expected. Furthermore geriatric hospitals often have many chronic bed-patients with incontinence of urine and faeces which tends to increase the number of yeast-induced diseases of the skin in particular Great attention is indeed paid in these nursing homes to the care of the skin. Prolonged administration of nutrients, intravenously or by nasogastric tube, increases the risk of yeast fungaemia in many inmates of these hospitals (*Kay et al* 1968 *Ashcraft & Leape* 1970 *Dennis* 1971)

MATERIAL AND METHODS

Sources of Specimens

The material studied included all the specimens (total 427) taken for fungal culture from patients in Myllypuro Municipal Nursing Home, Helsinki during the 3-year period July 15 1970 – July 15 1973. Myllypuro Nursing Home is a 220-bed institutional hospital for chronically ill old persons over 65 years of age and is owned by the city of Helsinki and supervised by the National Board of Medicine. It is situated in a modern 9-story brick building completed in July 1970. The hospital is divided into five identical 44-bed wards that are located one above the other on five floors.

Distribution of the specimens by subgroups is presented in Tables 1 and 2 At least the first sample from each patient—in some cases more—was taken before the possible institution of antimycotic treatment. The urine specimens were midstream specimens with the exception of a few catheterized urines.

The specimens were chiefly from patients in whom there was more or less reason to suspect a mycotic state because of some circumstance (such

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as onset of eczema of candidosis type presence of yeast cells in Gram-stained urine, prolonged treatment with broad spectrum antibiotics, etc.) Specimens were taken from a total of 155 patients with a mean age of 78 years. There were 118 females and 37 males. The greater part had sustained cerebral apoplexy or were affected with senile dementia many had cardiac failure and/or chronic urinary tract infection.

Fungal Cultures and Identification of Yeasts Species

The specimens were usually taken in the early morning and placed untreated into a sterile sample dish which usually could be forwarded within a few hours to the fungus laboratory of the Department of Serology and Bacteriology of the University of Helsinki. Primary cultures of the specimens were made in the laboratory on at least the following culture media: malt agar Mycobiotic agar Sabouraud agar Sabouraud broth, and Sabouraud-sp agar (contains penicillin and streptomycin to depress bacterial growth) Primary cultures of a large part of specimens were made also on Kimmig and Dixon agar and many were furthermore grown on certain other media. The specimens were usually cultured without previous treatment with the exception that a part of each sputum sample was first homogenized with pancreatin and some faeces samples were suspended in physiological saline. Every specimen was cultured both in test tubes and on dishes, and at room temperature 30° C and 37 C. Pure cultures were prepared of the isolated fungus strains. Identification of the yeast strains was done according to the principles presented in "The Yeasts" (*Lodder* 1970) Assimilation tests of most strains were made with 28 carbohydrates in liquid medium (prepared from Bacto-yeast nitrogen base) in which the fungus strains were allowed to grow at room temperature during a minimum of 20 days, but usually for a longer time.

RESULTS

The results as concerns yeasts in the fungal cultures are seen in Tables 1–3. A total of 188 specimens, or 44.0 per cent, yielded growth of yeasts. From the 427 specimens studied, 212 yeast strains belonging to 11 species were isolated among these were 112 strains of *Candida albicans* 45 of *Torulopsis glabrata* and 17 of *Candida parapsilosis*.

Candida albicans

The predominant yeast obtained in the cultures was, as could be expected, *Candida albicans* (Fig. 1) the incidence of which is the total specimen material was 26.3 per cent (52.8 per cent of isolated yeasts, Tables 1 and 2) It was most common in sputa from which it was isolated in 64 1 per cent of specimens. This rate is in good agreement with that encountered in a similar group of debilitated patients in whom onset of respiratory tract infections was rather frequent and who were affected to a large extent also with lung diseases proper (*Kahanpää* 1972) On the other hand the frequency of *Candida albicans* in the oral cavity and pharynx, 32 1 per cent,

TABLE 1 *Total Growth of Yeasts in Fungal Cultures by Specimen Groups and Growth of Candida albicans*

Specimen group	Cultivated specimens, total No	Specimens, positive for yeasts %	Isolated yeast strains, total No.	*Candida albicans* Total strains No.	Incidence in specimens %
Skin	130	41.5	62	33	25.4
Morning sputum	39	66.7	32	25	64.1
Oral cavity and pharynx	28	53 6	17	9	32.1
Vagina	16	50.0	9	5	31.3
Faeces	88	37.5	40	24	27.3
Urine	110	46.4	52	16	14.5
Blood	16		–		
Total	427	44.0	212	112	26.3

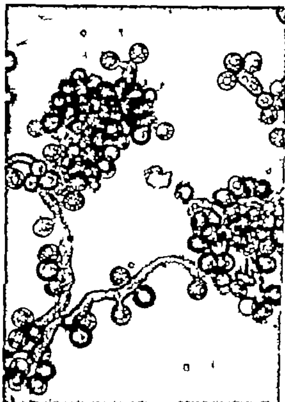


Fig 1 Typical chlamydospores of *Candida albicans* grown on corn meal agar × 500.

nence of faeces. Two of the aged patients, however were considered to have clinical candidosis of the urinary tract. *Candida albicans* repeatedly grew very abundantly from the urine and also direct microscopy of fresh specimens showed yeast cells and, in one of the two cases, also pseudohyphae. Neither patient had bacteriuria but both had a long history of pyuria and albuminuria. The increased frequency of and mild smarting on micturition that had troubled them for months disappeared during antimycotic treatment.

Torulopsis glabrata

The yeast fungus ranking second in frequency in the whole specimen material was *Torulopsis glabrata* with an incidence of 21 2 per cent of all isolated yeasts (Table 2) This supports the opinion that *Torulopsis glabrata* is increasing in prevalence in human specimen materials (*Bodey* 1966, *Kahanpää* 1972) It was very common now in the urine samples. This figure is slightly misleading, however since the group included two patients with urinary torulopsosis caused by *Torulopsis glabrata* from whom a number of urine specimens were taken during treatment. With this reservation the incidence of *Torulopsis glabrata* in urine (14 7 per cent of examined patients) was just barely lower than that of *Candida albicans* (16 0 per cent of examined patients) and accordingly agreed well with the observation by *Jansson* (1963) that *Torulopsis glabrata* is second in frequency of the yeast species found in urine series studied in Finland.

Although *Torulopsis glabrata* occurs mostly as an innocent saprophyte, it merits serious attention. Furthermore, according to the present author's earlier results, cited above, it is more common in the older age groups, particularly in association with lung diseases. Year by year there is an increasing number of reports in the literature on opportunistic infections by *Torulopsis glabrata* (*Hahn et al* 1968 *Lees et al.* 1971) In the present patient series, too this yeast was the causative agent not only in the two cases of urinary tract infection mentioned but quite obviously also in a case of stomatitis and one of colpitis.

Other Yeasts

The percentage distribution of yeast strains other than *Candida albicans* and *Torulopsis glabrata* varied between 8.0 per cent and 0.3 per cent (Table 2) Third in frequency was *Candida parapsilosis* which grew from specimens from the skin in particular Also in the light of the present material it seems that *Candida tropicalis* is somewhat more common in patients of advanced age than in the Finnish population in general (*Jansson* 1962 and 1963 *Kahanpää* 1972) The demonstration of *Pityrosporum ovale* and *orbiculare* is based on the use of Dixon agar for the greater part of this specimen material. It can also be mentioned that not a single strain of *Cryptococcus neoformans* was found

DISCUSSION

Infections caused by yeasts are common in geriatric hospital patients and affect especially certain cutaneous areas and the oral cavity. These infections may furthermore be highly resistant to treatment and have a tendency to recur (e.g. submammary or perianal candidosis of bed-confined diabetics). Oral yeast infections in elderly patients are often associated with the use of dental prostheses, but other forms, such as true thrush also occur (*Lehner* 1967, *Salo & Hirvonen* 1969). In the light of the present patient series, yeast infections of the urinary tract seem to be more common among old than among young persons (*Schönebeck & Ansehn* 1972). The possibility of e.g., the genitals and the lower respiratory tract being affected should also be kept in mind in these cases.

Although superficial yeast infection often presents a clear-cut clinical picture, omission to carry out a fungal culture may occasionally cause considerable difficulty in the treatment of these superficial infections, not to speak of the deep mycoses. Possibly with the exception of certain special instances, microscopic examination of a non-cultured specimen is not a substitute for fungal culture, though it does add to the information obtained from the latter. Sensitivity determinations of yeast strains have also begun to be considered helpful in many cases (*Bodenhoff* 1969). Likewise, the importance of a closer diagnosis of yeasts is being increasingly recognized (*Ruth* 1973).

Of major importance is the differentiation from other yeasts of the most common causative agent of yeast infections, *Candida albicans*. With a high degree of probability this is possible by means of e.g., such simple laboratory findings as the presence of chlamydospores (Fig. 1) and pseudo-germ tubes (*Kahanpää* 1972, *Ruth* 1973). On the other hand, it is worth noting for instance that nearly half of the yeast strains in the present work were strains other than *Candida albicans*. These other strains may include unexpected ones, some even of considerable clinical importance. Unless a closer identification is made in such cases, the causative agent of for example, cryptococcosis may escape recognition or treatment for a presumed yeast infection will be given when it is actually a question of usually a saprophytic species of *Saccharomyces* or *Pichia*. In fact, even among the about 80 species of *Candida* only some seven or eight are definitely known to cause human yeast infection (*Hurley* 1967, *Gentles & La Touche* 1969, *Kozinn et al.* 1969).

In consideration of the above the classification of yeasts into only two groups, i.e. *Candida albicans* and other yeasts, cannot be considered adequate. On the other hand an exact species classification of all the yeast strains isolated in a laboratory is impossible in practice, rather costly and even unnecessary from the clinical point of view. It should therefore be the aim to try to pick out, by the means available and in quantities possible in the individual laboratory, yeasts of major clinical importance such as *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis*, *Cryptococcus neoformans*, *Trichosporon cutaneum* and *Torulopsis glabrata*. Unfortunately often it may not be possible to *confirm* the identifications without the use of a broad scope of yeast identification systems such as those used in the present study and without the assistance of a laboratory specialized in this work.

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BACTERIOPHAGE TYPING OF *MYCOBACTERIUM RANAE (FORTUITUM)* THE USE OF UNADAPTED AND ADAPTED PHAGES IN THE DEVELOPMENT OF A TYPING SYSTEM

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Mycobacterium ranae has been used as an experimental model to investigate the feasibility of developing phage typing system for the subdivision of a mycobacterial species. Typing by means of unadapted phages was of limited value whereas, for one group of strains, a highly discriminative set of adapted phages was produced. The eleven phages used in the study were divisible into four groups, one consisting of three phages from pseudolysogenic strains. Two other groups have been named the *Ter* and *P aso* groups, respectively. Two hitherto undescribed phages have been isolated and designated Mx1 and Mx2.

Epidemiological studies on *Mycobacterium tuberculosis* have been somewhat limited by the lack of methods for the intraspecific typing of this species. Cell-wall agglutination techniques (*Schaefer* 1965, 1967) which have proved of considerable value in the study of the distribution of *M. avium* (*Pierning et al.* 197?) are not applicable to *M. tuberculosis* owing to the roughness of the strains. Phage typing which is of undoubted value in the study of *Staphylococcus aureus* and *Salmonella typhi*, has been used for the subdivision of *M. tuberculosis* (*Bates & Fitzhugh* 1967, *Tokunaga et al.* 1968) and applied to epidemiological problems (*Baess* 1969, *Bates & Mitchison* 1969). However in these studies the number of discriminative phages, and therefore the number of phage types, was rather limited.

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Phage typing of a bacterial species may either be performed by using a number of naturally occurring phages with different host ranges or by taking a single phage of limited host range and adapting it onto a large number of alternative host strains. The first principle is used for *Staph. aureus* and the second for the Vi phage typing of *S. typhi*. In the latter system, some phages show mutational changes, some show host-controlled modifications and others show a combination of these two types of variation (*Bernstein & Wilson* 1963).

It was therefore decided to investigate the feasibility of developing a phage typing system based on one or both of the above principles for a mycobacterial species. *Mycobacterium ranae (fortuitum)* was used as a model for these studies because this species has recently been subjected to detailed investigations of its serological and biochemical

TABLE 1 *Strains of* M. ranae (fortuitum) *used in the Study*

Collection No.	Original designation	Source or reference	Association with phages
4	NCTC 8573	*Wells et al.* (1955)	Lysogenic for Ø Mx2
5	NCTC 2006	*Wilson* (1925) Terrapin strain	
33	G.U.	*Beck* (1965)	Carrier of Ø BK5
81	NCTC 2891	Type strain of *M. ranae*	
108	16575	Dr *J. Marks* Cardiff	
109	18519	Dr *J. Marks* Cardiff	
487	8.69/750	Prof *F. Gatti* Zaïre	Propagator for Ø 13A
491	491/70	Middlesex Hospital	
576	2076	Prof *S. Pattyn* Antwerp	
577	2042	Prof *S. Pattyn* Antwerp	
630	ATCC 23021	Dr *G. Kubica* U.S.A.	
632	ATCC 23025	Type strain of *M. peregrinum*	
650	ATCC 23041	Dr *Desmyter* Zaïre	Carrier of Ø Mx1
676	1811	Prof *S. Pattyn* Antwerp	
679	1900	Prof. *S. Pattyn* Antwerp	
680	1954	Prof. *S. Pattyn* Antwerp	Propagator for Ø Mx2
681	2040	Prof *S. Pattyn* Antwerp	
690	1297/70	Middlesex Hospital	
799	19	Dr *M. Magnusson*, Copenhagen	
800	20	Dr *M. Magnusson* Copenhagen	
802	31	Dr *M. Magnusson*, Copenhagen	
807	931	Dr *M. Magnusson* Copenhagen	
843	516	Dr *M. Magnusson*, Copenhagen	Propagator for Ø BK4
844	ATCC 6841	Type strain of *M. fortuitum*	
859	ATCC 23029	Dr *G. Kubica* U.S.A.	Carrier for Ø BK6
864	SN 703	Dr *I. Tarnok* Germany	Propagator for Ø BO4 BO7 and BO16
870	T 120	Dr *Takeya* Japan	Propagator for Ø C3
RO	–	Mutant of strain no. 108	
SA1	–	Mutant of strain no. 108	
SA2	–	Mutant of strain no. 108	

variations (*Stanford* & *Gunthorpe* 1969 *Pattyn et al.* 1974)

In a previous study (*Grange* & *Nordström* 1973) a correlation between lysis by phage BK4 and inositol utilization in *M. ranae* was described. This paper reports the comparative host ranges of eleven mycobacteriophages and modification of host range by multiple adaptation of one selected phage.

MATERIALS AND METHODS

Organisms

The collection numbers and origins of the strains used in the study are shown in Table 1. The additional strains Ro, SA1 and SA2 were obtained as mutants of strain no. 108 (*Grange* 1973). The strains were examined by agar-gel immunodiffusion (*Stanford* & *Beck* 1968 *Stanford* & *Gunthorpe* 1969) and grouped according to the five serotypes of the species (*Pattyn et al.* 1974). They were tested for inositol utilization by the modified carbohydrate-nitritase row (*Grange* & *Nordström* 1973).

Bacteriophages

Phage BK4 isolated from soil near Copenhagen (*Baess* & *Weis Bentzon* 1969) was supplied by Dr *I. Baess*.

Phage BK5 (*Baess* 1971) and phage BK6 were isolated from strains 33 and 859 respectively.

Phages BO4 BO7 and BO16 all isolated from soil in northern Germany were supplied by Dr *I. Tarnok*.

Phage C3 isolated from the soil of a chicken-run in Japan by Takeya, was supplied by Dr *H. Engel*.

Phage *Minetti* was supplied by Dr *Gino Castelnuovo*. This phage was originally obtained as a host-range variant of phage *Phlei* (*Penso* & *Ortali* 1949).

Phages Mx1 and Mx2 were isolated by the authors from strains 650 and 4 respectively see below.

Phage 13A was obtained by the adaptation onto strain no. 487 of phage 13 which was isolated from soil in India and propagated on a strain of *M.*

smegmatis (*Sundar Raj* & *Ramakrishnan* 1970)

The host strains and propagator strains of the phages are shown in Table 1

Propagation of Phages and Phage Typing

The phages were propagated on the host strains listed in Table 1 The methods for phage propagation, titration and typing have been described previously (*Grange* & *Nordström* 1973) In this study the highest dilution producing confluent plaques on the propagator strain was used as the routine test dilution (RTD) Typing was performed using RTD 10xRTD 100xRTD and 1,000xRTD

Adaptation of Phages onto Alternative Host Strains

Phage C3 50 ml quantities of nutrient broth were inoculated with the serotype I strains (except the lysogenic strains 4 and 859) and incubated for twenty four hours at 32° C. 0.2 ml of a suspension of phage C3 was added to each bottle and re-incubated for a further 48 or 72 hours After passage through a 0.22 μ membrane filter 2.0 ml of the filtrate was added to a further 24-hour culture of the new host strain. After fifteen such passages, the final culture filtrate was examined for lytic activity on the new host strain and the lytic titres were determined (see Table 3) Subsequently the phage C3 variants were re-adapted onto the

TABLE 2. *Lytic activity of the Eleven Mycobacteriophages on the Twenty-eight Strains of* Mycobacterium ranae

Strain	Serotype	INOS. MCNR	BK4	BO4	BO7	BO16	BK5	BK6	Mx1	C3	Min	Mx2	I3A
4	I	+	+	+	C	—	—	—	—	—	—	—	—
81	I	—	—	—	—	—	—	—	(+)	—	—	—	—
491	I	—	—	C	C	+	—	—	—	—	—	(+)	—
576	I	—	—	—	—	—	—	—	—	—	—	—	—
676	I	—	—	—	—	—	—	—	—	—	—	—	—
679	I	—	—	—	—	—	—	—	—	—	—	—	—
680	I	—	—	—	—	—	—	—	—	C	C	C	—
859	I	—	—	—	—	—	—	C	—	—	—	—	—
681	I	+	—	—	—	—	—	—	—	—	—	—	—
799	I	+	C	C	+	C	—	—	C	—	+	—	—
800	I	+	C	C	C	C	—	—	(+)	—	—	—	—
843	I	+	C	C	C	C	—	—	(+)	—	++	—	—
844	I	+	C	C	C	C	—	—	—	—	—	+	—
Ro	I	+	C	C	C	C	—	—	—	—	—	(+)	—
108	II	+	C	C	C	C	—	—	—	—	—	—	—
109	II	+	+	+	—	+	—	—	(+)	—	—	—	—
577	II	—	C	C	C	C	—	—	—	—	—	—	—
690	II	+	C	C	+	C	—	—	—	—	—	—	—
487	III	+	++	C	+	C	—	—	—	—	—	—	C
5	IV	+	+	+	—	+	—	—	+	—	—	—	—
33	IV	+	—	—	—	—	C	—	+	—	—	—	—
650	IV	+	—	—	—	—	—	—	C	—	—	—	—
630	V	—	—	—	—	—	—	—	—	—	—	—	—
632	V	—	—	—	—	—	—	—	—	—	—	—	—
802	V	—	—	—	—	—	—	—	—	—	—	—	—
807	V	—	—	—	—	—	—	—	—	—	—	—	—
8A1	V	—	—	—	—	—	—	—	—	—	—	—	—
8A2	V	—	—	—	—	—	—	—	—	—	—	—	—
864	I			C	C	C							
868	I										C		
870	I									C	C		

* *Key*
INOS, MCNR = Inositol utilization detectable by the modified carbohydrate-nitratase test.
Phage lysis C = confluent lysis at RTD
++ = confluent lysis at 10 RTD
+ = confluent lysis at 100 RTD
(+) = *confluent lysis at 1000 RTD*

original host strain no. 870. After six passages on this strain the re-adapted phages were examined for host range on the serotype I strains.

Phage I3 A suspension of phage I3 propagated on *M. smegmatis* was added to strains 108, 487 33 and 632 by the above method. Adaptation only occurred onto strain no. 487 This adapted phage was designated I3A.

Phages BK4 and BO4 Attempts were made to adapt these phages onto strain no. 81 (the type strain of *M. ranae* NCTC 2891) by the above technique.

Detection of Pseudolysogeny

During initial phage typing studies, pour-plates containing the test strains were inspected for the spontaneous appearance of plaques.

RESULTS

Isolation of Phages Mx1 and Mx2

Two hitherto undescribed phages were found. Phage Mx1 was isolated from plaques appearing spontaneously on strain no 650 (ATCC 23041) Culture filtrates from strain no. 4 (NCTC 8573) produced plaques on strain 680 The phage isolated from these plaques was designated Mx2.

Bacteriophage Typing

The twenty eight strains of *M. ranae* are listed in Table 2 according to immunodiffusion serology The table shows the inositol-utilizing activity of the strains and the results of typing by the eleven phages. Four phages, BK4 BO4 BO7 and BO16 showed a very similar ,but not identical, host range. Three phages, BK5 BK6 and Mx1 all appear as spontaneous plaques on their host strains, indicating pseudolysogeny (*Baess* 1971) These phages have a restricted host range. Phages C3 *Minetti* and Mx2 have restricted host range, but all showed lysis of strain no. 680 at RTD Phage I3A lysed strain no. 487 (serotype III) only Subsequently a further serotype III strain was found (collection no. 677) and was also lysed at RTD by phage I3A. Eighteen of the twenty eight strains were lysed by one or more of the bacteriophages. The ten strains not lysed by any of the phages included four of the serotype I strains and all the type V strains.

Adaption of Phages

Attempts to adapt phages BK4 and BO4 onto the inositol negative strain no. 81 were consistently unsuccessful. Phage I3 was adaptable onto the serotype III strain as discussed above.

Phage C3 was adaptable onto some of the serotype I strains. Table 3 shows that, with

TABLE 3 *Lytic Activity of the Host-range Variants of Phage C3 on the Serotype I Strains**

Test strain	Phage C3 on alternative host strains 870	680	576	843	676	81	679	Lytic titre PFU/ml	Inositol MCNR	Lysis by phages BK4 BO4, BO7 BO16
870	C	+ +	—	C	C	+	—	10^{10}	—	—
680	C	C	+	C	C	C	—	10^{8}	—	—
576	—	—	C	—	—	—	—	10	—	—
843	—	—	—	C	+	+	—	10^{7}	+	+
676	—	—	—	—	C	—	—	10^{7}	—	—
81	—	—	—	—	—	C	—	10^{8}	—	—
679	—	—	—	—	—	—	C	10^{8}	—	—
491	—	—	—	—	—	—	—	nil	—	+
681	—	—	—	—	—	—	—	nil	+	—
799	—	—	—	—	—	—	—	nil	+	+
800	—	—	—	—	—	—	—	nil	+	+
844	—	—	—	—	—	—	—	nil	+	+
RO	—	—	—	—	—	—	—	nil	+	+

*Key Lytic titre = the lytic titre of the filtrate from the final adapting passage used for typing.

TABLE 4 *Lytic activity of the Host-range Variants of Phage C3 Re-adapted onto the Original Host Strain (No. 870)**

Test strain	Phage C3 re-adapted from 680	576	843	676	81	679	Original Phage C3	Phage Minetti
870	C	C	C	C	C	C	C	C
680	C	C	C	C	C	C	C	C
576	—	—	—	++	—	—	—	—
843	++	+	C	++	C	C	—	++
676	—	++	—	++	—	+	—	—
81	—	—	—	—	—	—	—	—
679	—	—	—	—	—	—	—	—
491	—	—	—	—	—	—	—	—
681	—	—	—	—	—	—	—	—
799	—	—	—	—	+	—	—	+
800	—	—	—	—	—	—	—	—
844	—	—	—	—	+	—	—	—
RO	—	—	—	—	—	—	—	—

Key As in Table 2.

the exception of strain no. 843 phage C3 only adapted onto the strains that were neither lysed by phages BK4 BO4 BO7 and BO16 nor showed inositol-utilizing activity. The host ranges of the adapted phages are shown in Table 3.

The variants of phage C3 were all found to be re-adaptable onto the original host strain no. 870. The host ranges of the re-adapted phages, after six passages on the original host strain, are shown in Table 4.

DISCUSSION

Two new phages are described in this paper. Phage Mx1 was isolated from plaques spontaneously appearing on strain no 650. Phage Mx2, isolated from strain no. 4 is of restricted host range and might have remained undetected save for the coincidental inclusion of a sensitive indicator strain, no. 680, in the studies. *Bönicke* (1969) has suggested that lysogeny in the mycobacteria is probably much more common than suspected but often remains undetected due to a lack of suitable indicator strains. Certainly strain no. 4 (NCTC 8573) is a well known organism (*Wells et al.* 1955) and is issued by the National Collection of Type Cultures for use in the testing of disinfectants. Nevertheless lysogeny has not been previously observed in this strain.

The eleven phages used in the study were divisible into four groups. The first group included phages BK4 BO4 BO7 and BO16 all of which were isolated from soil in northern Europe. These phages showed a very similar host range and a close correlation with inositol utilization as described previously for phage BK4 (*Grange & Nordström* 1973). They did not, however lyse the two pseudolysogenic serotype IV strains and only lysed strain no 109 at 100 RTD. The latter strain produces deoxyribonuclease (personal observation) which is a property of some lysogenic mycobacteria (*Mankiewicz & Tamari* 1972) so this strain may exhibit superinfection immunity. Likewise, the anomalous results obtained with the typing of strain no 4 (lysogenic for phage Mx2) may be due to partial superinfection immunity. The four phages in this group have been named the *Tara* phages (*named after a* Tantric deity).

A second group of three phages, BK5 BK6 and Mx1 were all obtained from plaques which appeared spontaneously on their respective host strains indicating a pseudolysogenic carrier state. One of these

strains no. 33 carrying phage BK5 has been described in detail by *Baess* (1971) It is of interest that two of the three pseudolysogenic strains belonged to the rather uncommon serotype IV group

The third group contained phages C3 *Minetti* and Mx2 all of which showed restricted host ranges but possessed the common property of lysing strain no 680 at RTD These phages are, unlike the *Tara* group of diverse geographical origin phage C3 was isolated in Japan, phage *Minetti* originated in Italy and strain no 4 lysogenic for phage Mx2 was cultured from a facial abscess in Malta (*Wells et al* 1955) It is suggested that this group of phages should be known as the *Penso* group

The fourth group contained only one phage, I3A, which was a host range variant of phage I3 This phage only lysed the two serotype III strains and attempts to adapt phage I3 onto other serotypes of *M ranae* were not successful. Phage I3A is thus the only mycobacteriophage so far examined that has been found to be serotype-specific.

Although the results discussed above indicate some aspects of mycobacteriophage host interrelationships that merit further studies they do not form a satisfactory basis for a phage typing system for this species. It was therefore decided to investigate the possibility of developing a detailed phage typing system using several adaptations of a single phage of limited host range. Phage C3 was therefore adapted onto a group of serotype I strains although, for reasons that are not understood the phage did not adapt onto the strains that were either lysed by the *Tara* phages or utilized mositol, an exception being strain no 843 The results obtained by typing the serotype I strains with the set of adapted C3 phages somewhat resembled the Vi phage typing system for S. *typhi* (*Bernstein & Wilson* 1963) in that most of the adapted phages retained the ability to lyse the original host strain and strain no 680 in addition to their new host strains.

The unadapted phage C3 had the most restricted host range of the three phages in the *Penso* group. However after re-adaptation of the variants of this phage onto the original host strain, an expanded host range was observed as shown in Table 4 In this respect they resembled phage *Minetti* (also shown in Table 4 for comparison) which was originally obtained as a host range variant from a phage propagated on a strain of *M. phlei* (*Penso & Ortali* 1949) Thus in this group of phages adaptation does not appear to be due to simple reversible host mediated modification although the exact genetic nature of the observed changes are unknown.

Phage typing of Mycobacterium ranae might be of interest and value under certain uncommon circumstances, for example the investigation of an epizootic due to this species (*Darzins* 1952) However the results of these studies suggest an approach to the development of phage typing systems for mycobacterial species of greater clinical and epidemiological significance.

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MYCOPLASMOSIS EXPERIMENTAL PYELONEPHRITIS IN RATS

The Effect of Secondary Infection with Escherichia coli

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The concentration and persistence of *M arthritidis* in obstructed and unobstructed kidneys of rats after intracardial inoculation has been investigated. Inoculation of 10^8 c.f.u. *M arthritidis* giving a deposit of 10 c.f.u./g kidney tissue, depressed the resistance against *E. coli* so that secondary intravenous inoculation of 10^8 or 10^7 of *E coli* would be followed by severe acute pyelonephritis. These doses of *E. coli* could not provoke inflammation if given without primary mycoplasma inoculation.

In human pyelonephritis, *E coli* is frequently isolated in significant numbers ($> 10^5$/ml) from the urine (17) Furthermore it is possible under experimental conditions to produce pyelonephritis in rats by inoculation with *E coli* strains (8, 10 12) This indicates that this species of bacteria may play a prominent role in the development of pyelonephritis.

As demonstrated by several authors, among others (1 3 4 14) mycoplasmas occur frequently in the lower urinary tract of man and they are also in a few cases isolated from the upper urinary tract, but their pathogenic significance has not been proved (13 18) It was recently demonstrated in rats that a strain of *Mycoplasma arthritidis* was able to produce pyelonephritis in kidneys the ureters of which were ligated (16)

The purpose of this work was to investigate the effect of a secondary inoculation with *E coli* following a primary experimental infection with *M arthritidis*

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MATERIALS AND METHODS

Rats White Sprague-Dawley specific pathogen-free rats (male) weight 200–300 g were used.

Strain of Mycoplasma Strain 150 P 10 of *M arthritidis* the pathogenicity of which had been increased through passage in rats (6) was used for inoculation. The strain was cultivated in Hayflick's medium without penicillin and thallium-acetate. The culture was centrifuged in a Sorvall refrigerated centrifuge (RC 2B) at 34,000 x g for 30 min. The sediment was suspended in phosphate buffered saline (PBS) pH 7 4 and stored in volumes of 1 ml at –70 C (16)

Strain of bacteria. Strain 04 44/41 of *E. coli*, isolated from a patient with inflammation of the urinary tract was obtained from Statens Seruminstitut, Copenhagen. The strain was cultivated for 24 hours in 50 ml of brain-heart broth. The culture was centrifuged at 17,000 x g The sediment was resuspended in 50 ml NaCl 0.9 per cent and stored in amounts of 1 ml at – 0 C.

Inoculation. The rats were anaesthetized and the left ureter was ligated according to the technique described earlier (16) Immediately after this, 1 ml of mycoplasma suspension was injected intracardially Control rats were infected with 1 ml PBS. Twenty hours post inoculation (p.i.) the ureter

TABLE 1 *Determination of Colony Forming Units per Gram of Tissue in Obstructed and Unobstructed Kidneys at Different Intervals after Inoculation with 10^8 c.f.u. of* M. arthritidis

Time interval	Obstructed kidney c.f.u./g tissue	Unobstructed kidney c.f.u./g tissue
½ hour	7×10^3	10^3
	10^4	4×10^3
1 hour	10^4	5×10^3
4 hours	10^4	$< 10^2$
	3×10^3	< 10
24 hours	10^4	$< 10^2$
	5×10^5	$< 10^3$
48 hours	10^6	$< 10^3$
	5×10^4	$< 10^2$
72 hours	3×10^6	$< 10^3$
4 days	10^6	$< 10^3$
	10^6	$< 10^2$
9 days	4×10^6	$< 10^3$
10 days	3×10^6	$< 10^3$
	10^6	$< 10^2$
11 days	10^6	$< 10^3$
	10^6	$< 10^2$
	$< 10^3$	$< 10^2$
	$< 10^3$	$< 10^2$
13 days	10^6	$< 10^2$
	7×10^6	$< 10^2$
	$< 10^3$	$< 10^3$
	$< 10^3$	$< 10^2$

ligature was cut and removed. Six days p.i. the rats were inoculated in a tail vein with 1 ml of *E. coli* suspension. Control rats were injected with 1 ml 0.9 per cent NaCl.

Post mortem examination. Ten days p.i. the rats were euthanized and the kidneys removed aseptically. For quantitative cultivation of mycoplasmas and bacteria, 0.5 grams of kidney tissue was homogenized in 4.5 ml PBS using a Potter S homogenizer. Cultivation was performed on Hayflick's medium and blood agar.

For histological examination, kidney tissue was fixed in a buffered 4 per cent formaldehyde. Histological sections were stained with haematoxylin-eosin.

EXPERIMENTS AND RESULTS

Experiment I Determination of Mycoplasma Concentration in the Kidneys at Various Intervals p.i.

In order to evaluate the role of mycoplasmas in infections of the upper urinary tract it was necessary to obtain information about the distribution and persistence of the organism in the kidneys following intracardial inoculation.

The left ureter of 23 rats was ligated and 10 colony forming units (c.f.u.) of *M. arthritidis* were inoculated intracardially. The ligature was cut after 20 hours. At various intervals, the rats were euthanized and quantitative cultivating for mycoplasmas from both kidneys was attempted. The results are listed in Table 1.

One hour p.i., no difference was found in the number of mycoplasmas per gram of tissue in the two kidneys. Four hours p.i., no mycoplasmas were cultivated from the unobstructed kidney whereas the number of mycoplasmas in the obstructed kidney increased slowly till 48 hours p.i., whereupon it remained constant till approximately ten days p.i. After 11 and 13 days, the number of mycoplasmas was found to be less than 10^3 c.f.u./g tissue in 2 out of 4 of the rats sampled at these stages.

Experiment II The Effect of Ligation of the Ureter without Inoculation of Mycoplasmas or Bacteria

Four rats were injected with 1 ml PBS following the ureter ligation and six days later injected with 0.9 per cent NaCl.

The unobstructed kidneys were invariably sterile and without macroscopically or histologically demonstrable lesions.

The obstructed kidneys were also sterile with one exception where *Staph. albus* was cultured to the number of 10^7/g tissue. The kidneys were hydronephrotic, but no other lesions were seen either macroscopically or microscopically (Table 2).

Experiment III The Effect of Inoculation with M. arthritidis

Ten rats were inoculated with 10^8 c.f.u. of *M. arthritidis* and six days later injected with 0.9 per cent NaCl. *M. arthritidis* was cultivated from the obstructed kidneys to the number of 10^2–10^3 c.f.u./g tissue. *Staph. albus*

TABLE 2 *The Pathogenic Effect of Inoculation of Various Numbers of* M. arthritidis *and* E. coli *on the Obstructed Rat Kidney*

Exp	No. of rats	*M arthritidis* No. of c.f.u. inoculated	*E. coli* No. of bacteria inoculated	*M arthritidis* No. of c.f.u./g recovered from obstructed kidney	*E. coli* No. of bacteria/g recovered from obstructed kidney	Pyelonephritis
II	4	0	0	$< 10^3$‡	$< 10^3$	0
III	10	10^8	0	$< 10^3–10^4$	$< 10^3$	+
IV	8	0	10^7	$< 10^3$	$10^6–10^7$	0
V	3	10^8	10^4	$< 10^3–10^8$	$< 10^3$	+
	3	10^8	10^6	$< 10^3–10^8$	$< 10^3$	+
	3	10^8	10^8	$< 10^3–10^8$	$10^6–10^8$	+ + +
	14	10^8	10^7	$< 10^3–10^8$	$10^8–10^8$	+ + +

* 0 No macroscopically or histologically lesions.
 + : Minute focal inflammatory lesions.
 + + + Severe suppurative pyelonephritis.
‡ $< 10^3$: Lowest number tested.

was cultivated from the left kidney of two rats to the number of 10 /g tissue. *E coli* could not be cultivated Macroscopically the obstructed kidneys were found to be normal except for hydronephrosis. Histological examination revealed hydronephrosis in all obstructed kidneys and minute focal inflammatory lesions in the papilla of the kidneys from three rats (Table 2)

Experiment IV Effect of Inoculation with E coli

Eight rats were injected with PBS and six days later inoculated with 10^7 *E coli*. *E coli* was cultured from the obstructed kidneys to the number of 10^6–10^7/g tissue, and also *Staph aureus* was cultured from three of these to the number of 10^6/g tissue. *M arthritidis* was not cultured Except for hydronephrosis, the obstructed kidneys were found macroscopically and microscopically normal (Table 2)

Experiment V Effect of Combined Infection with M arthritidis and E coli

A total of 14 rats were inoculated with 10^8 c.f.u. of *M arthritidis* and 10 of *E coli*. The unobstructed kidneys were invariably sterile and without macroscopically or histologically demonstrable lesions.

M arthritidis was cultivated from the obstructed kidneys to the number of 10^4–10^8 c.f.u /g tissue. *E coli* was cultivated to the number of 10^7–10^8/g tissue. *Staph albus* was cultured from three kidneys to the number of 10^5–10^8/g tissue. Macroscopically the kidneys were moderately enlarged (hydronephrosis) wedge-shaped areas of inflammation being visible on the surface The pelvis was stuffed with pus. Histological examination revealed severe inflammation with neutrophilic leucocytes. The lesions were most pronounced under the renal pelvic epithelium, but progressed through the medulla and cortex to the surface. The glomeruli were normal. Large accumulations of neutrophilic leucocytes were found in the pelvis These lesions may be considered as a severe suppurative pyelonephritis (Table 2)

The pathogenic effect following secondary inoculation with lower concentrations of *E coli* was examined as well. Severe pyelonephritis was also produced by 10^8 of *E. coli*, whereas 10^6 and 10^4 of *E. coli* did not aggravate the minute mycoplasma lesions (Table 2)

DISCUSSION

Rats seem to be well suited for experimental studies of mycoplasma infections of the upper urinary tract, since they developed pyelonephritis if 10^8 c.f.u. of *M. arthritidis* was given after ligation of the ureter (16).

The lesions were not very severe despite the high dosage of inoculum and it was desirable therefore to determine whether mycoplasmas in small doses were able to produce lesions which could intensify the pathogenic effect of a secondary bacterial infection.

This happens to be the fact. A dose of 10^4 c.f.u. of mycoplasmas which resulted in an initial concentration of 10^4 c.f.u./g of kidney tissue was found to provoke a severe suppurative pyelonephritis if followed by an inoculation with 10^4 *E. coli*. These doses and a tenfold higher *E. coli* dose did not produce lesions in kidneys which had not been previously infected with mycoplasmas.

The synergistic effect of concurrent mycoplasmal and bacterial infection has been described earlier. In chickens, *M. gallisepticum* has been found to have a potentiating effect on secondary *E. coli* inoculation (5, 7) and the same effect of *M. pneumoniae* on the development of *Diplococcus pneumoniae* sepsis has been demonstrated (11).

A possible pathogenic connection between primary mycoplasma infection and secondary bacterial infection has been investigated by *Simberkoff & Elsbach* (15). They found that rat leucocytes lost their ability to phagocytose *E. coli* if the leucocytes were incubated previously in a suspension of *M. arthritidis* or *M. hominis*.

By cultivation for bacteria, *Staph. albus* was in the present study isolated from a few rats. However, this finding was not correlated to any particular experiment or experimental group. The occurrence of *Staph. albus* was not related to the occurrence of histological lesions and it has to be considered as a harmless contaminant either introduced during the process of ureter ligation or carried by the blood stream or ascended via the urinary tract to the hydronephrotic kidney.

These studies were carried out with *M. arthritidis* which in a few cases has apparently been isolated from man from different sites without any particular affinity to the urinary tract (2, 9). On the other hand, *M. arthritidis* has a well-known pathogenic effect in rats. The synergistic effect of *M. arthritidis* on *E. coli* infection may therefore be a rat specific phenomenon considering the relative species specificity of *M. arthritidis*. It should be determined whether the mycoplasmas of the human urinary tract (*M. hominis* and T mycoplasmas) possess the same potentiating effect.

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Burnet opines that immune surveillance is cell-mediated just as delayed hypersensitivity reactions, and incompatible graft rejection (*Burnet* 1970b)

This paper concerns the topic of immunological surveillance, and particularly some observations which are not immediately reconcilable with the concept as it now stands. If immune surveillance exists, it appears that it is dissociated from the normally accepted mechanism of cell mediated immunity in that mice of a constitution which allows acceptance of allo- and heterografts from closely and remotely related donors, and which therefore would be expected to be more prone to malignant disease, do not evidence a higher incidence of malignant disease.

The animal concerned is the mouse mutant *nude* (*Isaacson* & *Cattanach* 1962 *Flanagan* 1966) which displays congenital absence of the thymus (*Pantelouris* 1968) The observations stem from probably the largest colony of *nude* mice in existance over the four years from 1969 to 1972

MATERIALS AND METHODS

The Mouse Mutant Nude

The mouse mutant *nude* was first mentioned by *Isaacson* & *Cattanach* in 1962. Thereafter there was a lapse until *Flanagan* (1966) gave his detailed report on the animal. Phenotypically the mutant is distinguished by hairlessness, or by occasional sparse zonal hair growth. *Flanagan* studied the genetics of the mutation and showed it to be dependent on a recessive autosomal gene, *nu* located in linkage group VII (*Carter* & *Falconer* 1951) It was not until 1968 that *Pantelouris* discovered that the absence of hair was regularly associated with *absence of the thymus*. He also found that the *nude* mutant had very low leucocyte counts, and confirmed *Flanagan's* report that the life expectation of *nude* mice was short The animals are very susceptible to infection and Flanagan's animals usually die at 2-3 months of age with signs of wasting.

The similarity of this condition to that following neonatal thymectomy in mice (*Miller* 1961) encouraged one of us (J.R.) to set up a breeding colony of *nude* mice in the Pathological Anatomical Institute Kommunehospitalet, Copenhagen, Denmark (*PAI*) This was done on the assumption that the *nude* mouse might prove to be an alternative to the neonatally thymectomized mouse in the study of immune functions.

Since then ample experimental evidence has accumulated to show that the *nude* mutant is immunologically incompetent in its cell-mediated responses. This is most evidenced by inability to reject allografts and heterografts (*Rygaard* 1969, *Rygaard* & *Povlsen* 1969 *Pennycuik* 1971 *Wortis* 1971 *Povlsen* & *Rygaard* 1971 1972 and *Povlsen et al.* 1973) but also by extremely low antibody production (*Rygaard* 1969 *Wortis* 1971 *Kindred* 1971 *Pantelouris* 1971 *Pritchard et al.* 1973)

Breeding Conditions of the Mutant Nude

Breeding was begun at the PAI in December 1968 with two heterozygous pairs obtained from Drs. *R. C Roberts* & *D S Falconer* The Institute of Animal Genetics, Edinburgh, Scotland. The females carried the phenotypical marker *Trembler* (*Tr*) and the males, *Rex* (*Re*) both dominant autosomal genes of linkage group VII (*Carter* & *Falconer* 1951) From March 1969 a breeding colony has been maintained at the Laboratory Animals Breeding and Research Centre Gl. Bomholtgaard (BOM) DK-8680 Ry Denmark, supervised by *C W Friis*. This latter colony was begun with mice from the PAI.

Various breeding schemes have been used in parallel at the PAI and BOM since 1969 (*Rygaard* 1969 *Rygaard* & *Friis* 1973) and observations from the two centres are considered together The genetic background of the animals concerned, apart from *nu* gene homozygosity has altered somewhat over the years, but this seems to have been without effect on the particular observations of this study and it therefore ignored.

Initially male heterozygous carriers of the *nu* gene were crossed with non-inbred NMRI/BOM_f females, obtained from Gl Bomholtgaard. This part of the material comprises about 10 per cent. At present the gene is under transfer to three inbred strains of mice BALB/c/A/BOM_f C3H/bi/BOM_f and C57/BL/6/BOM_f About 70 per cent of the *nudes* in this study have a BALB/c background (50-96 per cent of the genome) while C3H *nudes* and C57/BL/6 *nudes* comprise each, about 10 per cent the percentage of the genome of the inbred strains being 50-93 in each group.

The Observation Group from PAI

At the PAI approximately 2 400 *nudes* were bred during the four years period 1969-1972. Of these about 400 underwent thymus grafting or transfer of thymus or spleen cell suspensions at the age of 4 weeks. In some 30 per cent, this attempt at reconstitution of the immune responses failed as judged from subsequent experiments.

None of these animals is included in this present study

Thus observations at the PAI were made in 2,000 *nu de* mice, treated in various ways not supposed to have any reconstitutive effect on the immune mechanisms by skin grafting or challenge with soluble and insoluble antigens. Some 1 000 were used for heterotransplantation of human malignant tumours (*Rygaard* & *Povlsen* 1969) *Povlsen* & *Rygaard* 1971 1972 *Povlsen*, unpublished, *Povlsen et al.* 1973) No mouse showed any sign of metastates which might otherwise have caused difficulty in interpretation, and the whole group is included in the study Finally 200 *nudes* were untreated. In some instances blood was taken for haematological studies, otherwise animals were left undisturbed for their life span. The mean observation period for mice in the PAI-group was four months, from birth to sacrifice r spontaneous death

The Observation Group at BOM

A total of 8,800 *nudes* were bred at BOM in the four years period under study All of these were untreated, the mean observation time being three months from birth until sale to various users. A group of 900 nudes (93 per cent NMRI genome) was observed for 7 months, their whole life span.

Total Observation Time

At the PAI the total observation time was 8000 months (2000 mice observed for an average of four months each mouse) This is the equivalent of approximately 670 years of mouse life.

At BOM the corresponding figure was 2725 years,

$$\frac{(8{,}800 \times 3) + (900 \times 7)}{12}$$

and the summed time from both centres, thus, 3395 years, or the equivalent of the whole lif span of approximately 1700 untreated healthy laboratory mice.

Living Conditions

The mice at PAI are kept at room temperature of 27 C ± 1 C relatn humidity 55 per cent ± 5 per cent. The rooms are ventilated with filtered air exchanged 8 times per hour

At BOM room temperature is 24 C ± 1 C, air exchange 15 times per hour while the relative humidity is as the PAI.

In both places Makrolon® cages, type II are used. Feed pellets with mineral and vitamin additives are produced and autoclaved at BOM Sterile wood granulat is used for bedding, and tap water is supplied *ad lib*

Breeding and keeping at BOM is conducted under strict spf-conditions behind a personnel barrier while at PAI only one of three animal rooms has a personnel barrier The husbandry methods in both centres, and their effects on breeding and keeping are described in detail elsewhere (*Rygaard* & *Friis* 1973)

Methods of Observations

Untreated mice were scrutinized, at minimum, twice weekly and animals used in experimental studies were scrutinized much more frequently

Signs of spontaneous disease changes in skin appearance development of hunchback, lack of growth etc.—were looked for particularly tumours of the skin, and primary or secondary lymphadenopathy

Mice at the PAI which displayed wasting, genital or anal prolapse or other spontaneous disease conditions were killed and autopsied by one of the authors, as were mice sacrificed at the termination of experiments or for post mortem study Occasional autopsies were performed by other qualified personnel.

At BOM the autopsies were performed by a veterinary surgeon or by an experienced laboratory assistant, and apart from mice examined for the above mentioned reasons, a number of normal *nudes* were sacrificed and autopsied as a routine in the control program.

Mice were eviscerated, and heart and lungs, stomach, gut, kidneys and genitalia were scrutinized. Lymph nodes of the axillary and inguinal regions were exposed for inspection, as were mesenteric and mediastinal nodes.

Frequent random histological controls were made in both treated and untreated animals, t spontaneous death, sacrifice, or as part of the control program. Tissues were fixed in formalin, embedded in paraffin wax, and 7 μm sections were cut for staining

RESULTS

Malignant change was not observed in any animal. The reticulo-endothelial tissues, heart, lungs, stomach gut, genitalia, soft tissues, bone, and central and peripheral nervous tissues were always clear and there were no metastatic deposits from undetected primary tumours.

One benign growth was found a papilloma of the skin in a *nude* mouse of NMRI-back ground (approximately 75 per cent of NMRI genome) reared at BOM. Histological study confirmed papilloma of the skin with no suggestion of malignancy

In some *nudes* a percentage reducing from about 5 to 1 in the course of the study tumourous swellings of one or both periorbital and/or parotid regions were observed These could appear at any age, but were more frequent in the older mice. Macroscopic and microscopic examination showed abscess formation of the lacrimal, Harderian and parotid glands with no sign of malignancy Bacterial cultures made at BOM showed that the responsible organism was *Staphylococcus albus* Nonspecific inflammatory changes in the uterine horns were also found. The histology of these findings is described elsewhere (*Rygaard* & *Poulsen* in press) Occasionally cystic changes in lymph nodes (*Rygaard* & *Poulsen* in press) may mimic tumours.

DISCUSSION

The results of the observations reported herein are unambiguous No spontaneous malignancies appeared in the immunologically deficient nude *mice during the period of this study*

The implications of this negative observation must be considered in the light of the nature of the immune defect displayed by the *nude* mouse. *Pantelouris'* finding of the congenital absence of the thymus (1968) has been confirmed by anatomical and functional studies in many laboratories which are not invalidated by a subsequent report from the same author (*Pantelouris* & *Hair* 1970) of the finding of a tissue aggregation which might be a solely epithelial rudiment of the thymus The *nude* is deficient in those immune responses ordinarily ascribed to an intact thymus.

Wortis et al. (1971) demonstrated that normal thymocyte precursor cells are present in *nude* mice by the finding that *nude* bone marrow can restore peripheral lymphocytes, and repopulate the thymus and thymus-dependent areas (*Parrott et al.* 1966) in sublethally irradiated normal mice. Normal fetal liver implantation in *nudes* however does not lead to lymphocytic infiltration of the structure supposed to be a thymic rudiment. The *nude* displays marked leucopenia, primarily a lymphopenia as mentioned before, and will readily accept skin allografts and heterografts, most significantly heterografts of human malignant tumours. Apart from this manifestation of defective cell mediated immune response, defective humoral antibody production has also been demonstrated by study of the number of plaque forming cells (*Jerne* & *Nordin* 1963) after immunization with sheep red blood cells, and by various assays to detect antibody formation in response to antigen challenge. The deficiency state thus lies between those described by *DiGeorge* (1965) and *Nezelof et al.* (1964) not totally corresponding with either

At this stage it can be summarized that:

1) the mouse mutant *nude* is deficient in cell mediated immune responses, and in some humoral responses, and
2) the animal accommodates malignant tissue

References given so far concern only human tumour heterografts (man to mouse) but we have found that the *nude* will also accommodate murine malignant tissue, plasmacytomas and sarcomas, and a number of hybrid cell lines (obtained from *Dr G Klein* Department of Tumour Biology Karolinska Institutet, Stockholm, Sweden) All these grow well in the *nude* A further significant finding is that malignant change can be induced in the *nude* by the injection of 20-methylcholanthrene (*Poulsen*—to be published) "De novo" malignancy has, thus, been observed in the *nude*

A major consideration in the context of this article is the relationship between occurrence of spontaneous malignant change in man and mouse, and age. Modifications of this relationship in patients under or after immunosuppressive therapy and in patients with immune deficiency states, are also important.

In general, malignant change in man is largely manifest later in adult life (see e.g.

Dorn & Cutler 1959) but after immunosuppressive therapy the age/malignancy relationship may be supplanted by a time-of therapy/malignancy relationship.

Penn & Starzl (1972) summarized the incidence of *de novo* malignancies in transplant recipients. Extracting from the records of the informal registry in Denver Colorado, they reported the results of observation of 9131 patients with renal, and 189 patients with cardiac homografts. Ninetythree patients developed 95 malignancies. Among these patients are 366 who underwent transplantation surgery and subsequent immunosuppressive treatment in Denver Colorado who were particularly closely followed by the authors. Eighteen of them—4.9 per cent—developed malignant disease.

The interval between transplantation and the appearance of tumours varies a great deal, but it is striking that six of the patients in the total series evidenced tumours within 2 months of transplantation, two of them within one month. The average time of tumour appearance after transplantation was 30 months. It is clear that cancer development is enormously speeded up by the given therapy be it the introduction of the graft, the immunosuppressive therapy or the combination The age of patients developing tumours is striking—the average age 35 years (range 8–64 years)

In immune deficiency states in man a similar independence of age is striking (*Good* 1970) and all the immune deficiency states studied appear to be linked with an unexpectedly high incidence of cancers. It is noteworthy however that more than 90 per cent of the patients of both categories do not develop cancer This point will be considered later

While malignancies arising after immunosuppression are both epithelial and lymphoid in origin, there is a predominance of lymphoid malignancies in patients with immune deficiency states (*Good* 1970) This latter observation and the lymphoid malignancies arising in patients in connection with immunosuppressive therapy may be explained by a primary dysfunction of lymphoid tissue or by a specific challenge to lymphoid cells resulting from the immunosuppressive therapy

Spontaneous cancers in mice usually occur between the ages of 8 and 15 months, but earlier in mice which have undergone neonatal thymectomy and particularly if these animals are exposed to carcinogens Milieu has proven to be of considerable significance. Mammary carcinoma incidence increases rapidly as the number of mice per cage is reduced (*Andervont* 1944 *Albert* 1967) and *Andervont & Dunn* (1962) found malignant disease in 98 of 225 wild house mice at autopsy These findings suggest that environmental factors may be the whole, or a significant part, of the aetiology of cancer *Friis* (personal communication) opines that the microbiological milieu is significant mice raised under spf conditions are less prone to cancer than mice of the same strain raised conventionally

Whatever the causes of the observed events, the evidence reviewed herein indicates that there are similarities between spontaneous and induced immune deficiency states in man and mouse. Immune deficiency is associated with increase in incidence and earlier occurrence, of malignant disease in both species.

The vast majority of the *nude* mice which are the object of this report were reared under spf conditions, but even so and despite their short life span it is quite remarkable that no *nude* mutant evidenced malignancy

It is possible that, although *nude* mice are immunologically deficient, this deficiency does not include the part of the immune system which is employed in the generation of malignant cells in the lymphoid organs of patients with immune deficiency disorders or undergoing immunosuppressive therapy This is purely hypothetical, and fails anyway to account for the epithelial tumours which occur in such patients.

It could also be that the *nude* is rather an analogue of the 90–95 per cent of patients with immune disorders, primary or secondary who do not develop cancers. This majority

of patients evidences that cancer in the immune deficient remains the exception rather than the rule and—as is the case with graft recipients—that it is possible to coexist with an accepted graft without developing cancers.

This reversal of the argument well justified from the figures, makes the postulate of immunological surveillance unnecessary. Cancer could be caused solely by exogenous oncogenic agents—chemical or microbiological—effective in the absence of an "ordinary" immune function, cell mediated and/or humoral. The absence of cancers in *nude* mice might then simply reflect the absence of exogenous oncogenic agents in the milieu described herein. Transfer of the *nu* gene to inbred strains with high tumour incidence (e.g. the AKR strain) might serve to clarify this point.

One possibility remains to be discussed as a possible explanation for the absence of spontaneous cancers in *nude* mice. According to the theory of *somatic mutation* (*Boveri* 1914, *Bauer* 1928) malignant cells develop spontaneously due to somatic mutations occurring *in the course* of cell divisions. In cell cultures the mutation frequency is about 10^{-6}. It is apparent that if the same number of potentially malignant cells are constantly generated *in vivo* there must be an active mechanism eradicating most of these cells even in the deficiencies under discussion.

If the incidence of potentially malignant cells is the same in mouse and man it can be calculated that some 2,500 mice are needed to equivalate with one man on a weight/weight basis (2,500 × 25 gms = 62.5 kgms). If this argument holds true the total observation period in our *nude* material would correspond to only slightly more than 16 months out of the life of one man's cell mass. *During this period it would be highly* improbable from the clinical data given here in that malignancies even if they might be initiated by mutation, would manifest themselves in the mouse population. But this comparison on a weight—time basis is evidently not valid. In an elegant argumentation *Dawe* (1969)extends the findings of *Andervont* & *Dunn* (1962) mentioned before of tumour incidence in wild mice to a calculation of the expected tumour incidence in the blue whale, the body weight of which is 100 tons. By comparing whale and mouse weight year units, he arrives at the result that the whale would have a 6.7 × 10^{7} greater probability of developing a neoplasm than the mouse. Before completing its first year of life every blue whale should have developed about 20,000 neoplasms. As *Dawe* points out, all are aware, that the incidence of neoplasia is not a simple function of protoplasm at risk per time unit.

Applying this argument to the present problem, one would expect a higher tumour incidence in mouse than in the same volume of man.

It is apparent, that if a high number of potentially malignant cells are generated in the *nude* mouse—and in man— *but not established* there must be a mechanism responsible for their removal and this mechanism *must be "specific" in the usual immunological* sense of the word. A non-specific system without self recognition would endanger normal cells. Again, if a surveillance system was non-specific, and mutations occurred spontaneously at random in various tissues and organs, its absence would entail a much higher incidence of multiple cancers than is observed in patients with immune deficiency disorders, and in general. *Penn* & *Starzl* (1972) found only two double tumours, which roughly conforms with expectation (*Watson* 1953).

If this viewpoint of specificity can be accepted, and assuming at the same time

(a) that a surveillance *mechanism* is active in the majority of patients with primary or induced immune deficiencies

(b) *that this surveillance* mechanism will allow the acceptance of foreign tissue grafts in the latter without a concomitant outbreak of malignancy and furthermore

(c) that the immunologically deficient *nude* mouse must be surveilled in some way in that it does not develop cancers, in spite of a total lack of cell-mediated re-

sponse—as witnessed by the frank acceptance of foreign grafts in untreated recipients,

it can be concluded that

immune surveillance—if existing—is a biological function of multicellular organisms, "immune" i.e. specific, in nature, but differing from cell-mediated and humoral immunity in the usual sense of these words. Surveillance deficiencies may coincide with deficiencies in cell-mediated and/or humoral immunity but only in a minority of these cases, possibly suggesting surveilance to be a more primitive function.

Regarding cell mediated and humoral responses as two distinct aspects of immunity surveillance—generally ascribed to the cellular part—is postulated to be a *third* aspect. It may seem worthwhile to try to delineate the latter from the two former—generally accepted—aspects.

The ultimate conclusion could be that immunological surveillance would not be needed on the whole because neoplastic changes could simply be rare events, arising from exogenous challenge due to contacts with carcinogens—viral or chemical.

The absence of spontaneous tumours in our present material might then just reflect the absence of carcinogens in the present milieu.

However plausible this explanation seems to the authors, our observation do not allow a more precise answer at the moment.

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of patients evidences that cancer in the immune deficient remains the exception rather than the rule and—as is the case with graft recipients—that it is possible to coexist with an accepted graft without developing cancers.

This reversal of the argument well justified from the figures, makes the postulate of immunological surveillance unnecessary. Cancer could be caused solely by exogenous oncogenic agents—chemical or microbiological—effective in the absence of an "ordinary" immune function cell-mediated and/or humoral. The absence of cancers in *nude* mice might then simply reflect the absence of exogenous oncogenic agents in the milieu described herein. Transfer of the *nu* gene to inbred strains with high tumour incidence (e.g. the AKR strain) might serve to clarify this point.

One possibility remains to be discussed as a possible explanation for the absence of spontaneous cancers in *nude* mice. According to the theory of somatic mutation (*Bovers* 1914 *Bauer* 1928) malignant cells develop spontaneously due to somatic mutations occurring in the course of cell divisions. In cell cultures the mutation frequency is about 10^{-8}. It is apparent that if the same number of potentially malignant cells are constantly generated *in vivo* there must be an active mechanism eradicating most of these cells even in the deficiencies under discussion.

If the incidence of potentially malignant cells is the same in mouse and man, it can be calculated that some 2,500 mice are needed to equivalate with one man on a weight/weight basis (2,500 × 25 gms = 62.5 kgms). If this argument holds true, the total observation period in our *nude* material would correspond to only slightly more than 16 months out of the life of one man's cell mass. During this period it would be highly improbable from the clinical data given here in that malignancies, even if they might be initiated by mutation, would manifest themselves in the mouse population. But this comparison on a weight—time basis is evidently not valid. In an elegant argumentation *Dawe* (1969) extends the findings of *Andervont* & *Dunn* (1962) mentioned before of tumour incidence in wild mice to a calculation of the expected tumour incidence in the blue whale, the body weight of which is 100 tons. By comparing whale and mouse weight year units, he arrives at the result that the whale would have a 6.7×10^7 greater probability of developing a neoplasm than the mouse. Before completing its first year of life every blue whale should have developed about 20,000 neoplasms. As *Dawe* points out, all are aware, that the incidence of neoplasia is not a simple function of protoplasm at risk per time unit.

Applying this argument to the present problem, one would expect a higher tumour incidence in mouse than in the same volume of man.

It is apparent, that if a high number of potentially malignant cells are generated in the *nude* mouse—and in man— but not established there must be a mechanism responsible for their removal, and this mechanism must be "specific" in the usual immunological sense of the word. A non-specific system without self recognition would endanger normal cells. Again if a surveillance system was non-specific, and mutations occurred simultaneously at random in various tissues and organs, its absence would entail a much higher incidence of multiple cancers than is observed in patients with immune deficiency disorders, and in general. *Penn* & *Starzl* (1972) found only two double tumours, which roughly conforms with expectation (*Watson* 1953).

If this viewpoint of specificity can be accepted, and assuming at the same time

(a) that a surveillance mechanism is active in the majority of patients with primary or induced immune deficiencies
(b) that this surveillance mechanism will allow the acceptance of foreign tissue grafts in the latter without a concomitant outbreak of malignancy and furthermore
(c) that the immunologically deficient *nude* mouse must be surveilled in some way in that it does not develop cancers, in spite of a total lack of cell-mediated re-

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AN IMMUNOFLUORESCENCE STUDY ON THE HUMORAL IMMUNITY TO *STREP 2A* IN RECURRENT APHTHOUS STOMATITIS

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The occurrence of humoral antibodies to *Strep 2A* in recurrent aphthous stomatitis (RAS) was investigated by titrating sera and by using a double-layer immunofluorescence staining method. The series comprised 24 patients with RAS and 24 persons without anamnestic or clinical signs of RAS. The distribution of endpoint titre in RAS differed significantly in relation to the distribution in the controls ($p<0.005$). No antibodies to a strain of *Streptomyces* and a strain of *Neisseria* used were demonstrated in RAS. The results indicate that *Strep 2A* hypersensitivity is a feature of RAS.

Recurrent aphthous stomatitis (RAS) (syn. recurrent aphthous ulceration, recurrent aphthous ulcers, recurrent oral ulceration, recurrent oral aphthae and recurrent Mikulicz's aphthae) is a disease of the oral mucosa. It is characterized by recurrent, burning, smarting, painful, single or multiple, erosive or fibrinopurulent, well-demarcated ulcerations with a peripheral red halo. The healing takes place without scar formation.

The aetiology of RAS is obscure. Microbiological and immunological investigations into the aetiology have dealt with the role of virus (*Blank et al.* 1950, *Stark et al.* 1954, *Ship et al.* 1961, *Sallay et al.* 1971 a, b, *Nász et al.* 1971), the possibility of an autoimmune mechanism (*Lehner* 1964, 1967 a, b, 1969 b, c, *Brody & Silverman* 1969, *Dolby* 1969, 1970) and the possibility of hypersensitivity to *Strep. sanguis* prototype 2A. (*Graykowski et al.* 1966, *Barile et al.* 1968).

The streptococcal hypersensitivity theory was not confirmed by *Francis & Oppenheim* (1970). They found impaired lymphocyte stimulation by *Strep 2A* in RAS, indicating immunological hyporesponsiveness to *Strep 2A*.

Recently *Donatsky & Bendixen* (1972) demonstrated by means of the leucocyte migration test (LMT) cellular hypersensitivity to *Strep 2A* in RAS.

To investigate the streptococcal hypersensitivity theory further, the purpose of the present investigation was to study by help of the immunofluorescence (IF) technique the humoral immunity to *Strep 2A* in patients with RAS.

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MATERIALS AND METHODS

The series comprised 24 patients (12 females, 12 males, age 17–69, average 36 years) with anam-

TABLE 1 *Reactions for Establishing Specificity of a Positive Strep 2A Immunofluorescence Staining*

Antigen	Antisera	Conjugate	Results
Strep. 2A	Phosphate buffered saline	RaAH Ig/FITC	Negative
Strep. 2A	Positive serum absorbed with Strep. 2A	RaAH Ig/FITC	Negative
Strep. 2A	Positive serum	RaAH Ig/FITC	Positive

nestic and clinically characteristic RAS. Twenty four persons (11 females, 13 males, age 20–60 average age 31 years) without any anamnestic or clinical evidence of RAS served as controls. On the day of examination 9 patients were in a period of remission. Fifteen patients were in a period of recurrence. Blood was collected from a cubital vein and the serum stored at −20 C. Humoral antibodies were detected by a *double-layer immunofluorescence* staining method.

Antigens

Strep 2A and strains of the species *Neisseria* and *Streptomyces* were used. The *Strep 2A* was the strain of *Strep sanguis* used by *Donatsky & Bendixen* (1972) in the leucocyte migration study The strain of *Neisseria* was chosen as a representative of the strains isolated from some recurrent aphthous ulcerations (*Donatsky 1972*) The strain of *Streptomyces* was chosen because the genus *Streptomyces* is not pathogenic or resident of the human body The strain of *Strep 2A* was subcultured and prepared as previously described (*Donatsky & Bendixen 1972*) The strains of *Neisseria* and *Streptomyces* were subcultered on horse blood agar and aerobically multiplied in 5 per cent horse serum broth by incubation at 37 C for 24–48 hours. The bacteria were killed by heating t 60 C for 30 minutes. The bacterial sediments were washed three times in 0.9 per cent NaCl and resuspended. These bacterial suspensions were stored at +4 C and used in all the investigation which was carried out in 4–5 weeks. Before use, droplets of all three bacterial suspensions were placed on slides and airdried.

Antisera

The first layers used in the IF-staining were titrated sera from patients or controls, Serum dilutions 1 1 1.2, 1·4 1.8 1 16 1.32 were used. If the sera still were positive at 1.32 further dilutions were made.

Conjugates

The conjugates used were rabbit antihuman heavy chain specific globulins conjugated with fluorescein isothiocyanate (FITC) The specificity was obtained by immunization with pure IgG or IgM followed by absorption with free light chains, and tested on monoclonal bone marrow cells. The rabbit antihuman IgG titre and the rabbit antihuman IgM titre were 100 (i.e. 1 ml conjugate absorbs 100 μg pure human IgG or IgM, respectively) The mean optical density ratio at 495 nm/280 nm was 0 66 with 0.50 as lowest and 0.95 as highest ratio. Unconjugated immunoglobulin molecules with optical density below 0.50 and conjugated immunoglobulins with optical density ratio above 0.95 were removed by ion exchange chromatography The conjugates were prepared and tested by Dakopatts, Ltd., Copenhagen, Denmark. The conjugates were diluted 1 10 and mixed in equal parts. That means a working titre of 1·20 of the rabbit antihuman IgG/FITC and the rabbit antihuman IgM/FITC in the mixture of conjugate used in all the experiments.

IF Staining and Microscopy

The IF staining method was performed by incubation the slides at room temperature in a moist chamber with dilutions of serum for 30 minutes, washed three times for 5 minutes in phosphate-buffered saline (PBS) at pH 7.2 and incubated for another 30 minutes with the mixture of conjugate. Before mounting in PBS at pH 7.2 with 10 per cent glycerol, the slides were washed again three times for 5 minutes.

The IF staining of the bacteria was done twice with each serum. Results were read as positive or negative. The fluorescence microscope set up was that used by *Dabelsteen & Rygaard* (1972) The magnification was 700 × using a 70x oil fluorite objective NA 1 15 and 8x periplan eyepiece. To ensure that the *Strep 2A* fluorescence staining reactions were specific, the experiments summarized in Table 1 were carried out. The absorption of the 48 sera with *Strep 2A* was carried out using diluted but strongly positive sera, i.e. if possible the sera were diluted to a titre two steps below the endpoint titre before absorption, but if the endpoint titres were too small, undiluted sera were used. The dilutions were mixed with equal parts of bacterial sediments for 2–3 hours at 21 C.

RESULTS

The distribution of the antibody endpoint titres against *Strep 2A* in 24 controls and 24

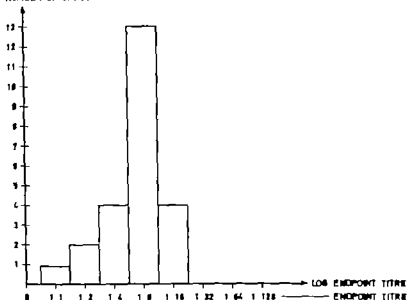


Fig 1 Histogram showing the distribution of humoral antibodies to *Strep 2A* in 24 controls. The endpoint titres were obtained by immuno-fluorescence staining.

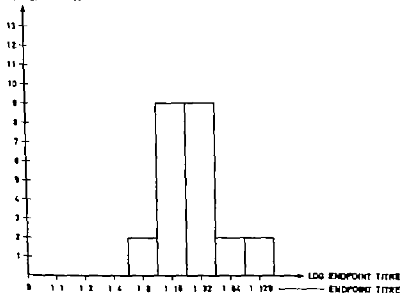


Fig. 2 Histogram showing the distribution of humoral antibodies to *Strep 2A* in 24 patients with RAS. The endpoint titres were obtained by immunofluorescence staining

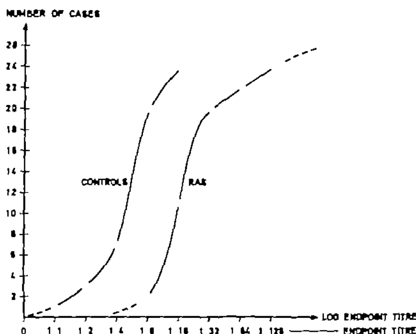


Fig 3 Cumulative distributions of the histograms in Fig. 1 and Fig 2. (Sign. – ● – ● – Controls, – O – O – RAS)

patients with RAS is shown in Fig 1 Fig 2 and Fig 3

The histograms and the cumulative distributions indicate that the controls and the patients form two different populations. The antibody titres of the controls and the patients ranged from 1 1 to 1 16 and from 1.8 to 1 128 respectively

Statistical analysis using the Mann Whitney U-test (*Siegel 1956*) shows that the mean value of the endpoint titre against *Strep 2A* in the group of controls is significantly smaller than the mean value of the endpoint titre against *Strep 2A* in the group of patients with RAS ($p < 0.005$)

The sera from 15 patients with recurrence at the day of examination showed endpoint titres ranging from 1 8 to 1 128. The endpoint titre in the sera from 9 patients with remission ranged from 1.8 to 1·64 The two groups showed no significant difference in the distribution of endpoint titres.

The two sera with the highest demonstrable antibody titres were female sera, but no significant difference in titre were demonstrable in relation to sex.

The control reactions with phosphate buffered saline as the first layer were all negative. The absorption with *Strep 2A* succeeded in all 48 sera since the slides in the following set up series all were negative

All the sera showed negative reactions to the strain of *Streptomyces* Two sera from controls showed slightly positive reactions to the strain of *Neisseria* using undiluted sera. After dilution 1 2 these two sera were negative. The sera from patients with RAS demonstrated no antibodies to the strain of *Neisseria*

DISCUSSION

In the present study it was possible by help of the IF-staining and the fluorescence microscope system used to demonstrate antibodies to *Strep 2A* in patients with RAS as well as in controls. The results showing a quantitative difference of the antibody titre to *Strep 2A* in the patients with RAS in relation to the controls indicate a humoral hypersensitivity to *Strep 2A* in RAS

This finding confirms that there may be

an association between RAS and *Strep 2A* which has previously been suggested by isolation of *Strep sanguis* from aphthous lesions (*Barile et al.* 1968 *Donatsky* 1972) by demonstration of intracutaneous reactions to *Strep sanguis* prototype 2A in RAS (*Graykowski et al.* 1966) and by *in vitro* demonstration of cellular hypersensitivity to *Strep 2A* in RAS (*Donatsky & Bendixen* 1972) The finding of immunological hyporeactivity to *Strep. 2A* in the lymphocyte transformation experiments (*Francis & Oppenheim* 1970) seems at a variance with the results indicating hypersensitivity However the impaired lymphocyte stimulation may be an epiphenomenon related to an antigen dose response lymphocyte paralysis or binding of immunocompetent cells in antigen containing tissue sites as suggested by *Francis & Oppenheim* (1970)

The variation in antibody titre in the patients in the IF-study present is similar to the distribution of migration indices (MI) in the leucocyte migration study (*Donatsky & Bendixen* 1972) The findings indicate a variance in the degree of sensitivity to *Strep 2A* The sera with the highest antibody titres were found in patients with recurrence. As each patient was tested only once the present study does not show whether the degree of humoral hypersensitivity is related to the recurrence of disease.

The specificity of the IF-staining was controlled by the negative results if phosphate buffered saline or absorbed sera were used as the first layer No specific antibodies to the strains of *Streptomyces* and *Neisseria* were found in RAS As to the strain of *Streptomyces* this result was expected since the genus *Streptomyces* is neither pathogenic nor resident of the human microbiological flora. The result obtained by the strain of *Neisseria* is remarkable, however as this bacteria was a representative of the *Neisseria* isolated from some aphthous ulcerations (*Donatsky 1972*) This negative result in detecting humoral antibodies to the strain of *Neisseria* used supports the possibility that the *Strep 2A* hypersensitivity in RAS could be specific

However if this theory is to be confirmed, further investigations using other bacteria especially other streptococci isolated from recurrent aphthous ulcers must be carried out. Another unclarified problem is whether the demonstrated immunity to *Strep 2A* is directly involved in the pathogenesis of RAS or merely a consequence of secondary infection of the recurrent aphthous ulcerations.

The conjugate used in the present study was a mixture of antihuman IgG and IgM. No further attempt was done to characterize the antibodies to *Strep 2A* As far as the presence of IgA antibodies to *Strep 2A* is concerned, this possibility was not studied. Therefore studies upon the characterization of antibodies to *Strep 2A* in RAS must be desirable.

Our studies and other studies on the influence of *Strep 2A* in the pathogenesis of RAS (*Barile & Graykowski* 1963 *Barile et al.* 1963 *Graykowski et al.* 1964 *Stanley et al.* 1964 *Graykowski et al* 1966 *Barile et al.* 1968 *Francis & Oppenheim* 1970) are not directly inconsistent with the auto-immune investigations (*Lehner* 1964 1965 a b 1967 a b 1968 1969 a, b c *Brody & Silverman* 1969 *Dolby* 1969 1970) or the virus investigations (*Sallay et al. 1971 a*, b, *Nász et al.* 1971) as demonstration of cross-reactions between *Strep 2A* and human oral mucosa (*Lehner* 1964 *Kramer* 1965) or between virus, *Strep 2A* and human oral mucosa would combine all the theories. To investigate the possibility of cross-reaction, experiments are in progress. Comparative investigations into the humoral and cellular immunity to human oral mucosa and to oral bacteria or virus isolated from aphthous ulcerations may be useful in the future explorations of the pathogenesis of RAS

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students all belonging to blood group A_1. All were classified as secretors by identification of blood group A substance in the saliva.

Blood grouping was performed at the Blood Grouping Department, Statens Seruminstitut, Copenhagen and the secretor status at the Institute of Forensic Medicine, Department of Serology Copenhagen.

The cells were obtained by scraping the buccal mucosa with a metal spatula after having removed the mucus and the superficial cell layers of the epithelium by means of a lancet. Care was taken to prevent saliva from coming into contact with the mucosa after the surface layer of the epithelium had been removed this procedure prevented contamination of cells with blood group antigen from saliva. The cells to be stained for blood group antigens were immediately fixed in test tubes containing 6.25 per cent glutaraldehyd. The cells to be stained for the cell coat material were fixed in test tubes containing 6.25 per cent glutaraldehyde fixative with 3000 p.p.m. ruthenium red. Both cell preparations were fixed at 4 C for one hour

Blood Group Antiserum

The anti-A serum was an anti-A test serum purchased from Hoechst® Frankfurt, Germany The serum has in a previous study (5) been shown to contain anti-A antibodies of the IgG class. In a light microscopical study (5) the titre of these antibodies in an immunoperoxidase staining of buccal mucosa from the four students under investigation has been estimated to approximately 1 128. In the staining reactions in the present study it was used in a concentration of 1:5.

Conjugates

The peroxidase conjugate was a rabbit antihuman IgG It was purchased as heavy chain specific from Dakopatts A-S, Copenhagen. Heavy chain specificity was obtained by immunization with pure IgG and afterwards by absorbtion with free light chains. The specificity was tested on monoclonal bone marrow cells, (Dakopatts®) The antibody titre was 320 (i.e. 1 ml conjugate absorbs 320 µg pure human IgG) and the peroxidase/protein ratio 1.20. On the basis of light microscopical studies (4) a working titre of 1 10 was chosen. The ferritin conjugate was a horse antihuman IgG antibody purchased from Cappel laboratories Inc., Downington, U.S.A. Antibody titre and conjugation degree were not available. The conjugate was used in a concentration of 1 10

Staining of Blood Group Antigen A

Blood group antigens on the oral epithelial cells were localized by means of a double layer staining technique with a human blood group antibody as the first layer and labelled antihuman IgG as the second layer (?)

Staining of the blood group antigens was carried out on suspensions of the epithelial cells. After fixation the cells were washed in phosphate buffer saline containing 6.8 per cent sucrose (PBS-S) pH 7.2 and centrifuged in an ECCD centrifuge at 3000 rev/min. The washing and centrifugation was repeated three times.

The cells were then incubated for a period of 30 minutes with anti-A serum at room temperature followed by another three washes in PBS-S and centrifugation. Then the cells were incubated with conjugated antibodies for 30 minutes at room temperature followed by washing and centrifugation three times in PBS-S. After the last centrifugation, the pellets were heated to 45 C and embedded in 3 per cent Bactoagar® Difco. For the stainings with the peroxidase conjugate, agar-embedded cells were incubated in a medium containing 5 mg of 3 3 diaminobenzidine tetrahydrochloride 10 ml of

TABLE 1 *Controls for Establishing Specificity of Immunoperoxidase and Immunoferritin Staining*

Blood group substance	Blood group antisera	Conjugate	Results
.	*Phosphate buffered saline.*	*Labelled antiglobulin.*	*Negative*
. .	Anti-A absorbed with A erythrocytes.	Labelled antiglobulin.	Negative
B	Anti-A with known reactivity	Labelled antiglobulin.	Negative
A*	Anti-A with known reactivity	Unlabelled antiglobulin followed by peroxidase reaction.	Negative
A	Anti-A with known reactivity	Labelled antiglobulin.	Positive
A	Anti-A with known reactivity	Unlabelled antiglobulin followed by labelled antiglobulin.	Negative

* For immunoperoxidase stainings only

0.05 M Tris-HCl buffer (pH 7.6) and 0.1 ml of 1 per cent H_2O for 30 minutes at room temperature. The agar blocks were then washed three times for five minutes in Tris-HCl (pH 7.6) cut into small blocks, and fixed in 2 per cent osmium tetroxide for half an hour. To ensure that the staining reactions were specific, control preparations were made: these are summarized in Table 1

Staining of Cell Coat

The material prepared for the investigation of the cell coat was after fixation washed for 5 minutes in PBS-S pH 7.2 and centrifugated at 3000 rev/min as described above. Following the last centrifugation the pellet was incubated in 10 per cent agar as previously described. The agar-embedded material was then cut into small blocks and post fixed in a 2 per cent osmium tetroxide containing 3000 p.p.m. ruthenium red.

To check on the reproducibility of the staining reaction all stainings were performed in duplicate.

Electron Microscopy

Following dehydration, the material was embedded in Epon. Semi-thin sections (1 micron) stained with toluidine blue were prepared for orientation using an LKB Ultrotome III ultramicrotome with glass knives. The thin sections (500-800A) were stained with uranyl magnesium acetate and lead citrate and examined in a Philips EM 200 electron microscope.



Fig. 1 One micron thick section showing exfoliated buccal epithelial cells from the superficial part of the epithelium. Toluidine-blue. × 600

RESULTS

The semi-thin sections demonstrated squamous epithelial cells at different stages of differentiation (Fig. 1). Some of the cells thus contained degenerating nuclei, others were anuclear. The cells were either isolated or lying in small groups in which intercellular relationship were maintained (Fig. 1). The following account deals mainly with isolated cells.

Labelling of the cell surface antigens with peroxidase conjugate revealed large amounts of electron-dense material along the outer part of the cell membranes (Fig. 2). The cell membranes were highly irregular and convoluted. The electron-dense reaction product was either evenly distributed along the cell membrane (Fig. 3) or had a patchy appearance. Frequently aggregates of electron-dense material were located between the small cytoplasmic villous projections. In a few cells only single clusters of electron-dense granules could be observed along the cell periphery (Fig. 4 & 5). In every section examined a few cells were found to be without reaction product.

In the small groups of squamous epithelial cells showing normal intercellular spaces, reaction product was observed intercellularly in a few sections only. Generally the intercellular spaces showed no reaction products (Fig. 6).

In all cells examined the reaction products were observed in intimate relationship to the outer leaflet of the trilaminar plasma membrane (Fig. 3 & 4). Most of the cells demonstrated an electron-dense cytoplasmic condensation along the inner aspect of the plasma membrane which identifies them as cells originating from the superficial layers of the epithelium (Fig. 4) and the cytoplasm was


3


4

dominated by loosely distributed electron-dense tonofilaments.

The labelling of the cell surface antigens with ferritin cojugate showed a pattern almost identical to the one described above. The reaction products appeared as discrete clusters of 2-8 small electron-dense ferritin particles along the outer surface of the plasma membranes (Fig. 7 & 8). In these sections, cells showing an irregular distribution of ferritin particles could also be observed, and cells without ferritin at the surface were seen, as described in the paragraph on the peroxidase labelling method.

All the epithelial cells stained with the ruthenium red method revealed a continuous electron-dense staining reaction along the surface of the cell membranes (Fig 9). At a higher magnification, the outer leaflet of the plasma membrane was seen to be coated evenly by the electron-dense granular deposit (Fig 10).

DISCUSSION

Exfoliated cells have been used instead of tissue sections in the present study in order to overcome problems associated with the diffusion of antibodies and conjugates.

The investigation has shown that it is possible at the ultrastructural level to demonstrate blood group substance A on exfoliated oral epithelial cells by means of immunoperoxidase as well as immunoferritin conjugates.

Fig 2 Part of superficial epithelial cell containing numerous tonofilaments (To). Along the cell surface large amounts of electron-dense material are observed representing the peroxidase reaction product. × 29,000

Fig. 3 The clusters of reaction product (arrows) are evenly distributed along the outer part of the cell membrane. × 47,000.

Fig 4 High magnification of the cell membrane showing a single cluster of peroxidase reaction product (arrow). An electron-dense cytoplasmic condensation is observed along the inner aspect of the plasma membrane (arrow head) indicating that the cell is from the superficial cell layers. × 95,000

It should be noted that the results obtained by the two techniques are almost identical. The reaction products and the ferritin particles indicating labelled antigens are found in intimate relationship to the outer part of the trilaminar cell membrane in the same position as the cell coat. The larger aggregates of peroxidase reaction product located between the cytoplasmic villous projections of the cells are believed be nonspecific reaction products collected in protected areas where washing is inadequate, and not being due to staining of blood group antigens.

The distribution of reaction product on the epithelial cell surface may be either even or patchy and in a few cells only very few reacting sites have been found.

It has been demonstrated (24 28) that an even distribution of blood group antigens as well as other cell surface antigens can change to a patchy distribution according to temperature and concentration of the conjugated antihuman antibody used for the staining reaction. This redistribution of surface antigens is introduced by the interreaction of antibody with the surface antigen and it is shown to be a metabolically dependent active process (28). In the present study the cells were fixed immediately after they were obtained from the oral mucosa and before the staining procedure was performed. Thus, the uneven distribution found on some of the cells can hardly be explained by "antigen redistribution"

Previous investigations (10 15 18 22) have revealed a fairly even distribution of antigen sites on erythrocytes along the entire cell surface of all the cells investigated. However it has recently been shown (17) that the development of red blood cells is accompanied by a significant increase in the number of antigen sites this may indicate a correlation between the number of antigen sites and the stage of cell differentiation. In previous light microscopical investigations of the oral epithelium (1 3) it has been demonstrated that the basal cell layer shows no blood group antigen reaction whereas this can be observed in the spinous cell layer. In the superficial

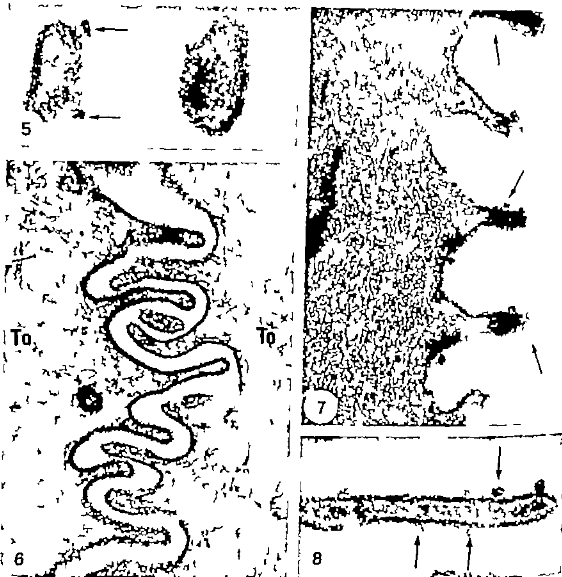


Fig 5 Cross-sectioned cytoplasmic projections with a few clusters of electron-dense peroxidase reaction product (arrows) × 82,600

Fig 6 Part of two epithelial cells showing normal intercellular relationships. Tonofilaments (To) No reaction product is found in the intercellular space. × 42,700

Fig 7 Labelling of the blood group antigens with ferritin conjugate. The reaction product is seen as clusters of small electron-dense ferritin particles (arrows) × 47,000

Fig 8 Cross-sectioned cytoplasmic projection with clusters of ferritin particles on the outer surface of the trilaminar cell membrane. × 74,000.

epithelial layers the reaction decreases towards the surface. This may reflect, as indicated in erythrocytes a correlation between antigen reactivity and stage of differentiation The variation in amount of reaction products on the cells observed in the present study might therefore be explained by the fact that we are dealing with cells in the last stages of the epithelial differentiation process.

The distribution of antigens in such oral

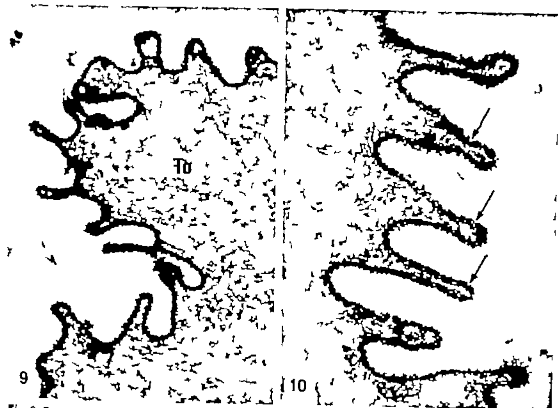


Fig 9 Part of an epithelial cell with tonofilaments (To) Along the cell surface a continuous layer of ruthenium red reaction product is seen. × 29,000.

Fig 10 Higher magnification of the cell membrane after staining with ruthenium red. The reaction product is seen as evenly distributed electron-dense granules (arrows) coating the outer part of the trilaminar cell membrane. × 47,500

epithelial cells as are showing an even distribution is similar to the distribution of HLA antigens on human lymphocytes (13) and of H 2 antigen on mouse thymocytes (23) However a direct comparison between the blood group antigen A and the histocompatibility antigen is not justified as they are associated with different carrier molecules on the cell membrane (16) Furthermore, the demonstration of antigen sites at the cell surface has proved partly to be dependent on the concentration of the conjugated antibody used (13) a reduced number of antigen sites being labelled parallel with dilution of conjugates. Furthermore, factors such as antibody titre and degree of conjugation of the antibodies are of importance for the results of the staining reaction. This makes a comparison of works by different authors even more difficult. In the present work, high concentrations of antibodies and conjugates were used in order to label as many antigen sites as possible.

The present work demonstrates that the distribution of blood group antigen A at the cell surface corresponds to the localization of the ruthenium red positive cell coat. The latter is more homogeneously distributed and appears, in contrast to the blood group substance, on all cells regardless of their stage of differentiation. The ruthenium red method is at the moment the best method for visualization of the cell coat (19) it is known, however that this method involves dehydra-

tion of the cell surface carbohydrates and may cause a collapse of these. This collapse thus representing an artefact may be responsible for the homogeneous appearance of the cell coat (*19*)

Partial or complete loss of blood group antigens A and B has been observed both in premalignant and malignant lesions developing from epithelium in which such substances are normally present (4 6 7 8 14 20) Previous studies have shown that derangements, mainly in the carbohydrate architecture of the cell surface, take place during malignant transformation (21) As the blood group antigen A is found in association with the cell coat, this opens up the possibility of using the blood group antigen A as a marker when cell surface changes during malignant transformation are to be studied.

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CORRELATION OF PHYTOHAEMAGGLUTININ-INDUCED LYMPHOCYTE TRANSFORMATION WITH CLINICAL MANIFESTATIONS OF SARCOIDOSIS*

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In vitro lymphocyte activation by phytohaemagglutinin was measured as ^{3}H TdR incorporation by buffy coat cultures from 90 patients with sarcoidosis. Neither the median response nor the geometric mean response in sarcoidosis differed significantly from those in controls. However when the series was divided into clinical subgroups, patients with hilar adenopathy and parenchymal pulmonary changes had significantly weaker responses. The PHA response was significantly higher in patients with erythema nodosum than in those without this symptom.

The impairment of cellular immunity in sarcoidosis is well documented (*Chase* 1966 *Scadding* 1967) In a number of diseases in which delayed hypersensitivity is impaired, the lymphocyte transformation induced by phytohaemagglutinin (PHA) is decreased (*Oppenheim* 1968) Many workers have observed a decreased response to PHA in sarcoidosis (*Hirschorn et al.* 1964 *Buckley et al.* 1966 1973 *Selroos* 1967 *Siltzbach et al.* 1969 *Jaroszewicz & Piotrowski* 1969 *Langner et al* 1969 *Kohout* 1971 *Rocklin et al* 1972, *Sharma et al.* 1971 *Topilsky et al.* 1972) but others have failed to find any difference in PHA induced lymphocyte transformation between controls and patients with sarcoidosis (*Cowling et al.* 1964 *Girard et al.* 1971) In the largest series studied, no significant difference in PHA induced ^{3}H TdR uptake was observed between 43 patients with sarcoidosis and 50 comparison subjects (*Girard et al* 1971) Because of these contradictory findings in the PHA response in sarcoidosis, PHA induced lymphocyte transformation was compared with the clinical manifestation of the disease.

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MATERIAL AND METHODS

Patients

The material consisted of 90 patients with sarcoidosis and 40 control subjects. The diagnosis of sarcoidosis was confirmed in all patients by a histologically confirmed positive Kveim test, and in most patients also by a lymph node or skin biopsy

According to the duration of the disease sarcoidosis was classified as acute (< 3 months) sub-acute (3–24 months) or chronic (> 24 months) The disease was classified as active in cases in which the lesions were progressive or in which the pulmonary lesions had remained unchanged for less than 1 year and as inactive when the lesions had not progressed during a period of more than 1 year According to the pulmonary changes the disease was classified into stages I II, and III (*Siltzbach* 1967) Patients had not been treated with steroids during a 6-month period before the in vitro tests were performed.

Controls were 23 healthy volunteers, and 17 patients with various mild non-allergic dermatoses, mainly toxic dermatitis of the hands, but otherwise healthy

All subjects were tested intradermally (Mantoux test) with 0.1 1 10 and 100 tuberculin units (TU) of purified protein derivative (PPD Statens Seruminstitut, Copenhagen) or until a positive skin reaction was obtained. In an intradermal Kveim test a Finnish Kveim preparation was used (*Putkonen* 1964)

Lymphocyte Cultures

20 ml of venous blood was withdrawn into heparinized (50 IU heparin per ml of blood) syringes and allowed to sediment for 1.5 hours at room temperature. The buffy coat was collected and centrifuged. The pellet was washed with Hanks balanced salt solution, centrifuged, and resuspended in medium RPMI 1640 with 20 per cent newborn calf serum (Orion, Pharmaceutical Co, Helsinki) The cell density was adjusted to 4×10^6 white blood cells (WBC) per ml. Cultures containing 2.5 ml were set up in 35 mm plastic Petri dishes (Falcon Plastics, Div Bacton Dickinson and Co) and incubated at 36.5 C in an atmosphere of 5 per cent CO_2 in air All cultures were set up in duplicate. PHA-P (Difco) was used in a final dilution of 1:250 which in preliminary experiments was found to give the optimal response in 3-day-old cultures. The same lot of PHA was used in all experiments, and stored in small aliquots at −20° C.

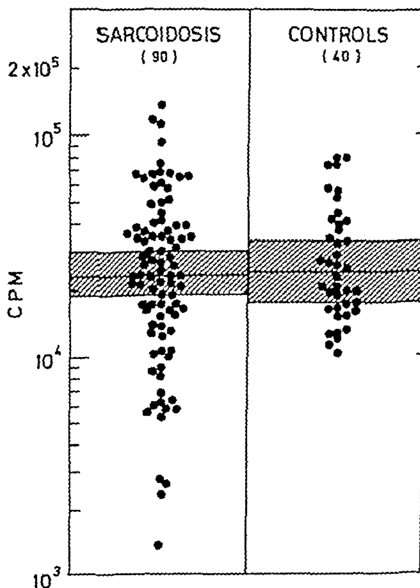
Measurement of Response

The PHA response was measured on the 3rd day One hour before the cultures were harvested, 1 μCi ^{3}H TdR (New England Nuclear Corporation, spec. activity 6.5 Ci/mMole) was added per ml of culture fluid. Incorporation of ^{3}H TdR into the acid-insoluble fraction was measured by liquid scintillation counting with a modification of the method described by Ilalsen et al. (1968) The entire procedure was carried out at 4 C and the reagents were chilled to the same temperature. 0.025 ml of 'carrier' (2 per cent bovine serum albumin in 10 per cent NaCl) followed by 3 ml of 5 per cent trichloroacetic acid (TCA) were added to 1 ml of cell suspension. After incubation for 30 min and centrifugation, the precipitate was washed twice with TCA, and dissolved at room temperature in 0.5 ml of 2 per cent NH_4OH. A 0.2 ml portion of the solution was spread on a filter pad (glass fibre paper GF/A, Whatman) previously placed in a scintillation vial. The open vials were heated at 110 C for 2 hours and 5 ml of scintillation fluid (0.3 g POPOP and 5 g PPO in one litre of toluene) was added. The radioactivity was counted in a Packard Model No. 3375 liquid scintillation counter Cell preparations for May-Gruenwald-Giemsa (MGG) staining were made with a Shandon cytocentrifuge to evaluate the viability of the cultures. In the presentation of the data for PHA-induced ^{3}H TdR incorporation, the uptake of ^{3}H TdR by control cultures without PHA was subtracted. 95 per cent confidence limits and Student's t test of paired comparisons were performed only in subgroups with more than 10 patients.

RESULTS

There were no significant differences in median response between patients with sarcoidosis and controls as measured by ^{3}H TdR uptake (Fig 1) When ^{3}H TdR uptake was expressed as the geometric mean response, the PHA-induced response was lower in the patients with sarcoidosis than in the controls, but the difference was not significant at the 5 per cent level (Fig 2) However several of the patients with sarcoidosis had a lower response than any of the control subjects (Fig 1)

To study whether the poor responders belonged to some sub-group of the patients with sarcoidosis, the PHA response was compared with various clinical manifestations of the disease, and with the sex, age and tuberculin skin sensitivity of the subjects (Fig 2) PHA induced ^{3}H TdR incorporation was significantly decreased in patients with hilar adenopathy and parenchymal pulmonary changes (pulmonary stage II) as compared with the controls. The PHA response was somewhat higher in patients with active erythema nodosum. The difference from the patients without erythema nodosum was significant, but neither group differed significantly from the controls. There was no significant cor



1 PHA-induced lymphocyte stimulation in cultures from 90 patients with sarcoidosis and 40 controls. The medians and 95 per cent confidence limits for the medians are shown.

relation of PHA induced lymphocyte transformation with other clinical manifestations or with Mantoux reactivity.

DISCUSSION

In the majority of the patients with sarcoidosis the PHA response was not decreased and the difference between the whole group of patients with sarcoidosis and the controls was not significant when the median responses or geometric means of ^{3}H TdR incorporation were used for comparison. Similarly in the largest series of patients with sarcoidosis previously studied, *Girard et al. (1971) did not find significant differences* in PHA induced ^{3}H TdR uptake between 43 patients with acute, untreated sarcoidosis, 30 healthy controls and 20 patients with acute allergic reactions.

To find an explanation for the contradictory findings in PHA induced lymphocyte

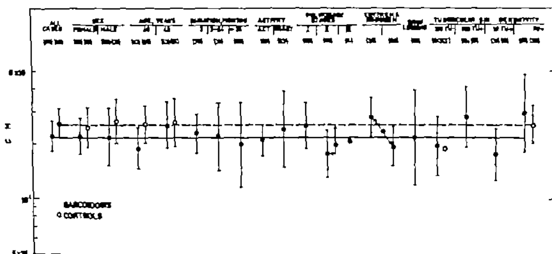


Fig 2 Comparison of the PHA induced lymphocyte stimulation to clinical manifestations of sarcoidosis. The results are presented as the geometric mean of cpm. 95 per cent confidence limits are shown for groups larger than 10. The number of cases is given in parentheses, Student's t test of paired comparisons was performed.
$p < 0.05$

transformation, the responses were correlated with clinical manifestations of the disease. Because this kind of comparison between many subgroups could cause unreliable results, Student's t test of paired comparisons were performed only in groups with more than 10 subjects. No significant correlation between PHA response and age or sex of the subjects was observed. A significant decrease in PHA response was found in patients who had hilar adenopathy and parenchymal pulmonary changes (pulmonary stage II). This group contained 44 of the 90 patients studied. In patients with active erythema nodosum, on the other hand, the PHA response was significantly higher than in those without this symptom. In many clinical studies the presence of pulmonary parenchymal changes has been reported to indicate a poorer prognosis in sarcoidosis, whereas erythema nodosum seems to point to a good prognosis (*Scadding* 1967 *Selroos* 1969 *Hannuksela et al.* 1970). Thus, the prognosis of sarcoidosis may be correlated to some extent with the PHA response.

The defect in cellular immunity in sarcoidosis is manifested by decreased skin test sensitivity (*Chase* 1966 *Scadding* 1967). *In vitro* tuberculin induced lymphocyte transformation was markedly lower in sarcoidosis than in controls and correlated with tuberculin skin test reactivity (*Hortmanheimo* 1974). No significant differences in PHA responses were found between patients with different degrees of Mantoux reactivity suggesting that there is no parallel between the decreases in PHA response and in tuberculin sensitivity.

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et al. (1968) and some studies done to determine the feasibility of this method in distinguishing between defects in ingestion and defects in intraleucocytic killing. Analysis of the results in terms of relationship between these two processes under normal conditions leads to a clear picture of the use and limitations of the method.

MATERIALS AND METHODS

Leucocytes. Venous blood was drawn into plastic syringes containing approximately 50 iu heparin (Leo, Copenhagen, containing 5 mg chlorbutol per ml) per ml of blood and mixed with 6 per cent dextran (Macrodex® Pharmacia, Copenh., Mw approximately 70,000 in 0.154 M saline) ratio blood to dextran 1:0.44 and left for sedimentation of erythrocytes, usually for 1 to 1½ hour at room temperature. The leucocyte-rich supernatant was withdrawn and the cells washed thrice in Hank's balanced salt solution containing 100 mg gelatin (Merck, Darmstadt, Germany) and 19.5 iu heparin without preservative per 100 ml. This basic culture medium was either adjusted to pH 7.45 by addition of 2.8 per cent sodium bicarbonate or to approximately pH 7.45 by addition of 10 per cent standard human serum (see below). Washing was carried out at low-speed centrifugation, 250 x *g*, for five minutes. The final cellular pellet was resuspended in this medium and adjusted to a concentration of 5 × 10^6 polymorphonuclear leucocytes per ml by haemocytometer counting.

Bacteria. Staphylococcus aureus phage type 42 E+ or strain 502A (kindly provided by Dr *K. Rosendahl* Statens Seruminstitut) were grown over night (approximately 18 hours) in meat infusion broth (Statens Seruminstitut). The bacteria were washed thrice in 0.154 M saline by centrifugation at 1,800 x *g* for 10 minutes, resuspended, and adjusted visually to a concentration ranging from 3 to 6 × 10^7 colony-forming units (CFU) per ml. This suspension was diluted ten-fold two times in culture medium to give a final concentration of 3 to 6 × 10^6 CFU per ml.

Standard serum. Freshly isolated serum from no less than 15 healthy blood donors was pooled and stored in small aliquots at −20 C.

Incubation mixtures. 0.8 ml of the bacteria suspension was mixed with 1.0 ml leucocyte suspension and 0.2 ml standard serum. If the leucocytes were washed in medium with 10 per cent serum, the mixtures contained 0.8 ml bacterial suspension, 1.1 ml leucocyt suspension, and 0.1 ml serum. The final leucocyte concentration was 2.5 × 10^6 polymorphonuclear leucocytes per ml with an approximately 1:1 ratio to bacteria and a serum concentration of 10 per cent. Incubations were carried out in 110 by 16 or 100 by 14 mm polystyrene tubes with polyethylene screw-caps (Nunc, Roskilde) at 35 C with end over end rotation at 20 rev/min. Duplicate tubes were set up for determination of total CFU and intracellular CFU.

Determination of total CFU. 0.5 ml of the incubation mixture was withdrawn at 2 and 4 hours and transferred to 4.5 ml distilled water followed in 5 minutes by vigorous pipetting to disrupt the cells. Ten-fold dilutions were made in 0.154 M saline and 1.0 ml of appropriate dilutions were mixed with 30 ml melted beef infusion agar (Statens Seruminstitut) at 48 C and poured into Petri dishes with a diameter of 7 cm. Colonies were counted after 48 hours and calculations of initial number of CFU in the sample were based upon the dilutions containing the highest number of colonies that could be counted, usually between 100 and 1,000 colonies per plate.

Determination of intracellular CFU. To separate tubes sodium benzylpenicillin, 100 iu per ml (Leo, Copenh.) and streptomycin sulphate, 100 µg per ml (Novo, Copenh.) in a total volume of 40 µl were added after 15 minutes incubation to kill remaining non-ingested bacteria. At 2 and 4 hours 0.5 and 1.0 ml incubation mixture was withdrawn and transferred to small glass-tubes, centrifuged at 250 x *g* for 5 minutes, and washed thrice in culture medium to get rid of the antibiotics. The final cellular pellet was resuspended in 1.0 ml distilled water followed in 5 minutes by vigorous pipetting. Serial ten-fold dilutions were made in 0.154 M saline and the rest of the procedure carried out as outlined above.

Determination of initial CFU. This was done by serial ten-fold dilutions and pour-plating of the initial bacterial suspension. In some experiments the effect of the antibiotics upon the bacteria in the absence of leucocytes was examined. Under these circumstances determination of surviving CFU was done after extensive washing and centrifugation at 1,800 x *g*.

Strict aseptic technique was ensured throughout the entire experimental procedure.

RESULTS

Stability of the test system. Addition of gelatin was found essential to secure a stable number of CFU. Omission of gelatin ca a tenfold decrease in CFU within one to t hours, which could be ascribed to agglutina tion as demonstrated in direct microscopy and in control tubes without rotation. In t presence of 10 per cent serum and absence o leucocytes the number of CFU remai constant for two hours, increasing therea

nearly ten-fold from two to four hours indicating growth following a lag phase. Viability of the leucocytes was repeatedly estimated by the trypan blue exclusion test which showed more than 98 per cent of the cells to be viable after four hours incubation. The total number of leucocytes was found constant by haemocytometer counting although some agglutination of the cells occurred. During incubation a slight increase in pH to approximately 7.8 regularly occurred. The functional capability of the cells as measured by reduction in total and intracellular CFU however was unaffected by variations in the pH of the medium between 7.32 and 7.82.

Effect of antibiotics. Incubation of bacteria alone with penicillin and streptomycin in these concentrations caused over 99.9 per cent reduction in CFU within one hour. Nearly the same effect could be achieved by streptomycin alone, whereas penicillin alone caused only a little more than 10 per cent reduction in four hours. This is in accordance with the findings of *Eagle* (1948). The effect of penicillin and streptomycin in combination was dependent upon two factors: 1) the presence of serum; without serum, the effect was about ten-fold less. This may be caused by an enhanced effect of streptomycin upon bacteria in the serum-containing medium which promotes rapid bacterial growth, and 2) the pH of the medium: at acid pH the killing effect was decreased whereas killing was most efficient at physiological pH. Under the experimental conditions of this method, effective elimination of non-ingested bacteria is thus achieved. On the other hand the inability of the antibiotics in these concentrations to influence the number of intracellular CFU has been amply demonstrated by the present technique (14) and related techniques (1, 8) in patients with a genetically determined defect in intraleucocytic killing: chronic granulomatous disease and in experimental models (21). This is furthermore supported by the present experiments shown in Fig. 4 and 6, where intraleucocytic killing was blocked by 1×10^{-4} M sodium azide (see below).

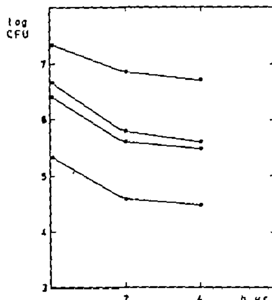


Fig. 1. Reduction in total CFU *Staph. aureus* with changing number of CFU initially present and a constant number of neutrophil granulocytes, 2.5×10^6 per ml. Mean of three experiments.

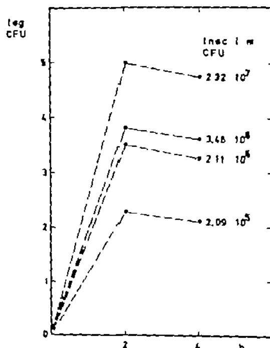


Fig. 2. Intracellular recoverable CFU *Staph. aureus* with changing number of CFU initially present and a constant number of neutrophil granulocytes, 5×10^6 per ml. Mean of three experiments.

Effect of serum concentration. A concentration of 10 per cent serum was found to be optimal for promotion of ingestion in the present system as evidenced by optimal reduction in total CFU This is quite consistent with previous findings applying related techniques (1 22)

TABLE 1 *Total and Intracellular Recoverable Colony-forming Units (CFU) of* Staphylococcus aureus *at Four Hours' Incubation in Relation to the Number of CFU Initially Present. Density of Neutrophils. 2.5 × 10⁶ per Ml*

Number of CFU per ml initially present	Total recoverable CFU	Intracellular recoverable CFU
2.26×10^7	20.8 per cent	
4.59×10^6	11 1 –	
2.30×10^6	12.9 –	
2.07×10^6	14.3 –	
2.32×10^7		0.25 per cent
3.48×10^6		0 12 –
2.11×10^6		0.068 –
2.09×10^6		0.055 –

* Colony-forming units *Staphylococcus aureus.*

Effect of density of bacteria. Fig 1 shows the effect of varying the number of initial CFU upon the number of total recoverable CFU Fig 2 showing the effect upon the number of intracellular recoverable CFU Over a 100-fold range in initial CFU a ... *y constant proportion can be recovered* ... *l and intracellular at 4 hours. The per* ... of recoverable CFU at 4 hours in these experiments are given in Table 1 These data are consistent with a proportional increase both in ingestion and intraleucocytic killing with increasing density of bacteria in *the suspension. The results shown in Fig 1* however would also be consistent with an increase in per cent ingested bacteria and a similar decrease in per cent killed but in that case a much higher percentage of initial CFU would be recovered intracellular at high density than at low density of initial bacteria *since increased* ingestion and decreased killing both would increase intracellular CFU

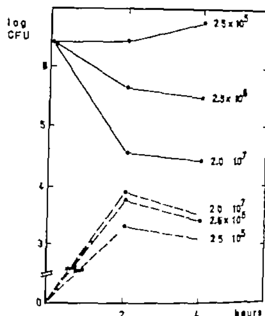


Fig 3 Recoverable total (——) and intracellular (– – –) CFU *Staph aureus* with changing number of neutrophil granulocytes as indicated in the figure. Mean of three experiments.

It is quite clear from the data presented in *Fig 2 that this is not the case*

Effect of density of leucocytes Fig. 3 shows that, over a nearly 100-fold range of concentration of neutrophils, the number of intracellular CFU after 4 hours varies only very little, whereas the number of total CFU is greatly increased with decreasing density of *neutrophils The percentage of recoverable* CFU at 4 hours in these experiments are given in Table 2 Since the data *presented in* Figs. 1 and 2 and Table 1 *indicate that the* number of bacteria to be killed is *directly* related to the number being ingested (i.e. a constant proportion of ingested bacteria are being killed over this range) it is to be expected that a higher number of bacteria are *being ingested when the density* of neutrophils is increased and an equally higher number are being killed, *resulting in a nearly* constant number of recoverable intracellular CFU The results presented in Fig 3 are *quite* in agreement with this. Still, as in the experiments shown in Fig 1 and 2 there is a slight tendency towards more effective killing if few bacteria are ingested The increase from 2 to

4 hours in total CFU at 2.5×10^6 neutrophils is caused by inadequate ingestion since growth of non ingested bacteria can be observed (Fig. 3)

TABLE 2. *Total and Intracellular Recoverable CFU Staph. aureus at Four Hours Incubation in Relation to the Number of Neutrophil Granulocytes Present in th Incubation Mixture*

Number of neutrophil granulocytes per ml	Total recoverable CFU*	Intracellular recoverable CFU
2×10^7	2.17 per cent	0.28 per cent
2.5×10^6	23.8 –	0.25 –
2.5×10^5	475 8 –	0 074 –

Colony-forming units *Staphylococcus aur s*

Effect of sodium azide Fig. 4 shows the effect of addition of sodium azide (Merck, Darmstadt, Germany) $1 \times 10^{-}$ M to the incubation mixture at the start of the reac tion. Reduction in total CFU is completely abolished which is clearly due to defective intraleucocytic killing as evidenced by the extremely high number of recoverable intra cellular CFU Contamination with viable non-ingested CFU was excluded in control experiments which showed that although kill ing of the bacteria with penicillin and streptomycin was less efficient in the presence of sodium azide still more than 99 per cent were killed within two hours. Other control ex periments using bacteria and sodium azide alone showed that azide at this concentration did not kill the bacteria within four hours, but multiplication was effectively prevented.

Effect of heat-inactivation of serum. Fig 5 shows that heat inactivation (56 C/30 min) of serum led to markedly impaired reduction in total CFU due to inadequate ingestion In fact, ample growth of non-ingested bacteria after 2 hours could be demonstrated. In spite of diminished ingestion, however the number of intracellular CFU is nearly the same in tubes with heat inactivated and in tubes with fresh frozen serum. These data parallel the experiments in which varying density of leucocytes was used (Fig 3 and Table ?)

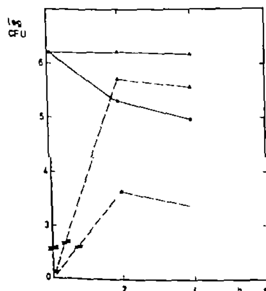


Fig 4 Recoverable total (——) and intracellular (---) CFU *St ph. au eus* with untreated neutrophil granulocytes (●) and treated with 1×10 M sodium azide (▲) Density of neutrophils 2. $\times 10^6$ per ml.

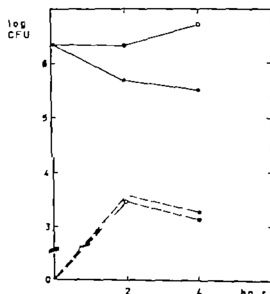


Fig 5 Recoverable total (——) and intracellular (---) CFU *Staph. au eus* in presence of heat inactivated (56 C/30 min) serum (○) and fresh frozen serum (●) Density of neutrophils .5 × 10^6 per ml. Mean of three experiments.

and further support the finding that relatively fewer are being killed within the observation time if ingestion of bacteria is reduced. Fig 6 shows that the difference between azide treated and untreated cells is somewhat larger in the presence of fresh frozen serum than in the presence of heat-inactivated serum. Since this difference will be an expression of the activity of intraleucocytic killing these findings could indicate an enhancing effect of heat-labile factors upon intraleucocytic killing These limited data, however do not allow any conclusions as to this important question This experiment also shows that ingestion is still possible in the presence of heated serum although to a reduced extent as seen from the number of intracellular CFU that could be recovered from the azide treated cells (open triangles)

Reproducibility of the method An estimate of the reproducibility of the method was done by running nine identically processed tubes from a single population of leucocytes for determination of intracellular CFU and nine for determination of total CFU The results are shown in Fig 7 which gives the mean and the range for each set of determinations. Good reproducibility of a single cell suspension is evident

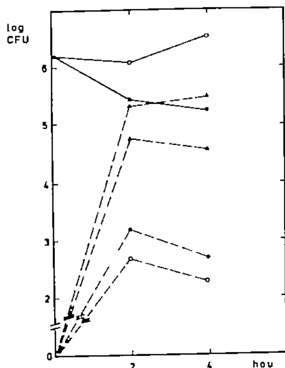


Fig 6 Recoverable total (——) and intracellular (– – –) CFU *Staph. aureus* in presence of heat inactivated (56°C/30 min) serum (○) and fresh frozen serum (●) ▲ indicates sodium azide (1×10^{-4} M) treated cells, and △ indicates azide-treated cells in presence of heat-inactivated serum. Density of neutrophils. 2.5×10^6 per ml.

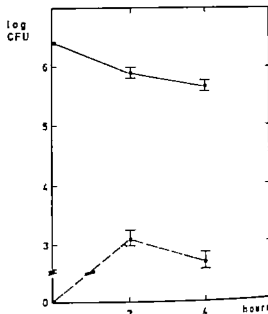


Fig 7 Mean and range of determinations of total (——) and intracellular (– – –) CFU *Staph. aureus* from nine identical incubation mixtures from one leucocyte population. Density of neutrophils. 2.5×10^6 per ml.

DISCUSSION

The present results indicate a near linear relationship between the rate of ingestion and the rate of intraleucocytic killing of this test organism by human neutrophil granulocytes. Results that would support these findings have been reported by others using related techniques. *Alexander et al.* (1968) reported variations in total CFU at 5 hours from 0.18 to 1 7 per cent over a range of $1\ 5 \times 10^5$ to 1.5×10^7 initial CFU per ml *Solberg* (1972 b) found a variation from 2.0 to 2.8 per cent at 2 hours over a range of 1×10^6 to 1×10^7 initial CFU per ml The present

variation ranged from 14.3 to 20.8 per cent over a range of 2.23×10^{6} to 2.94×10^{7} initial CFU per ml at 4 hours (Table 1). In contrast, variations in the density of leucocytes ranging from 5×10 to 5×10^{6} neutrophils per ml was found by *Alexander et al.* (1968) to give a variation in total CFU from 106 to 1.9 per cent at 4 hours which should be compared to the present values of 475.8 to 2.17 per cent total CFU over a range of 2.5×10^{7} to 2.0×10 neutrophils per ml (Table 2). The important point demonstrated in the present studies is, however, that over this range of density of neutrophils, the number of recoverable intracellular CFU did only vary from 0.074 to 0.28 per cent in spite of the large variation in total CFU (Fig. 3 and Table 2). Evaluation of data depicted graphically by *Solberg* (1972b), however, indicates a similar pattern of large variations in total CFU and minor variations in intracellular CFU with changing density of neutrophils. The apparent discrepancy between the present study and those cited above concerning total surviving CFU is readily explained on the basis of differences in density of neutrophils, system for mechanical mixing and possibly the leucocyte separation procedure (1, 22).

Biochemical studies of phagocytizing neutrophils offer a possible explanation of the relationship between rate of ingestion and rate of intraleucocytic killing indicated in the present studies. Both anaerobic glycolysis and respiration increase during phagocytosis but, while glycolysis seems to supply the energy for ingestion, effective killing is dependent upon normal oxygen consumption, hydrogen peroxide formation, and flow of glucose through the hexose monophosphate shunt (9). Several models linking these metabolic changes have been suggested (for extensive reviews see ref. 10 and 12). There is, however, general agreement that the burst of oxydative metabolism necessary for effective killing follows very rapidly after ingestion.

The present findings indicate a linkage of the metabolic changes associated with ingestion to those associated with intraleucocytic killing at a functional level. The degree of stimulation of metabolic activity may be determined by the number of bacteria ingested, and may again directly control the amount of energy released for intraleucocytic killing. Biochemical studies have in fact indicated a relationship between degree of particle ingestion and increment in oxydative metabolism (11). Furthermore, there is evidence that initial intraleucocytic killing may be limited by the amount of hydrogen peroxide supplied via respiration (13). The amount of stimulation of oxydative metabolism, however, may vary for the different micro-organisms to be ingested (17, 20).

The important point for the practical application of this and related techniques illustrated by the present studies is that increased ingestion does not lead to an increased number of recoverable intracellular CFU under normal conditions. Defects in intraleucocytic killing are readily disclosed by an increased number of intracellular CFU as demonstrated by the present and related techniques in patients and carriers of the neutrophil defect chronic granulomatous disease (1, 14). This is also in the present study demonstrated in the experiments with sodium azide treated cells (Figs. 4 and 6), and in other studies over the effect of experimental blockade of intraleucocytic killing (21).

Minor changes in the ingestion rate will hardly be detected by the present method. Since total surviving CFU at the employed density of neutrophils (2.5×10^{6} per ml) rarely exceed 20 per cent at 4 hours, it follows that at least 80 per cent are being ingested and even more when the density of neutrophils is increased (Fig. 3). Thus, depending upon the density of neutrophils, ingestion proceeds optimally or suboptimally due to mechanical promotion of contact between cells and bacteria. Furthermore, defective ingestion due to defects in random or directed migration of neutrophils (6, 19, 24) would hardly be detected. The experiments with heat inactivated serum (Figs. 5 and 6) show, however, that impairment of ingestion will in the present system be revealed by decreased

reduction in total CFU with minor or no alterations in intracellular CFU provided intraleucocytic killing proceeds normally. This is consistent with the earlier mentioned usefulness of the recording of total CFU for the detection of defective opsonization (15).

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EFFECT OF SODIUM AZIDE UPON NORMAL AND PATHOLOGICAL GRANULOCYTE FUNCTION

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Using an improved method for the evaluation of the phagocytic function of human neutrophil granulocytes in vitro the effect of sodium azide (NaN_3) upon the function of normal leucocytes and leucocytes from patients suffering from chronic granulomatous disease (C.G.D.) and from related carriers of C.G.D. has been investigated. 10^{-3} M NaN_3 causes marked inhibition of intraleucocytic killing of Staphylococcus aureus by normal leucocytes without interfering with the ingestion-phase. The determination of intracellular recoverable bacteria is highly sensitive in terms of demonstrating defective intraleucocytic killing and it is preferable to determination of total recoverable bacteria from the system. This has facilitated the detection of minor differences in intraleucocytic killing capacity between azide-treated normal leucocytes, C.G.D. leucocytes, and azide-treated C.G.D. leucocytes. Blocking of intraleucocytic killing with NaN_3 should prove a valuable tool in the investigation of the function of granulocyte bactericidal systems in clinical conditions.

The importance of precise evaluation of the capacity of human neutrophil granulocytes to kill ingested micro-organisms has been emphasized in later years by reports of congenital and acquired defects in intraleucocytic killing (2, 5, 7, 16, 22). The combined activities of ingestion and killing can be studied *in vitro* by incubation of the cells with a microbial test-organism followed by determination at prescribed intervals of total remaining viable organisms (intra- plus extracellular) by counting of colony forming units (CFU) (4, 17). Distinction between the ingestion- and the killing-effect requires, however, exact determination of intracellular CFU which in turn depends upon effective elimination of non-ingested CFU from the system. This can be accomplished with the aid of fastacting antibiotics which do not penetrate the leucocytes (8, 21). By simultaneous determinations of the recoverable total CFU and recoverable intracellular CFU alone, the efficiency of both the ingestion and the intraleucocytic killing can thus be determined with reasonable precision (15).

The number of viable intracellular CFU depends upon the rate of ingestion and the rate of intraleucocytic killing. The degree of intraleucocytic killing could therefore be evaluated more exactly if the total number of ingested CFU was determined simultaneously. This would be possible if intraleucocytic killing could be effectively prevented without interference with the rate of ingestion. For this reason the present studies were

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undertaken in which the effect of a metabolic inhibitor sodium azide, with a previously demonstrated blocking effect on intraleucocytic killing (12) was determined in an improved system recording both total and intracellular recoverable CFU (15).

MATERIALS AND METHODS

The technique employed is a modification of the method of *Alexander et al.* (1968) described in detail elsewhere (15). Briefly 2.5×10^6 polymorphonuclear leucocytes were mixed with *Staphylococcus aureus* strain 502A, in approximately 1:1 ratio of cells to bacteria in gelatinized, heparinized Hank' balanced salt solution in the presence of 10 per cent normal human pooled serum. Incubations were carried out in volumes of 2.0 ml at 35 C with end over end rotation at 20 rev/min. For determination of total CFU 0.5 ml reaction mixture was withdrawn at 2 and 4 hours, transferred to 4.5 ml distilled water for disruption of the cells followed by serial ten-fold dilutions and pour-plating. Colonies were counted after 48 hours incubation at 35 C. For determinations of intracellular CFU penicillin, 100 iu per ml, and streptomycin, 100 μg per ml, were added to separate tubes after 15 minutes' incubation. 0.5 and 1.0 ml were removed at 2 and 4 hours, respectively transferred to separate tubes followed by washing of the cells to get rid of the antibiotics, lysis in distilled water serial dilution, and pour-plating. Initial CFU was determined separately by serial dilution and pour-plating of the initial bacterial suspension. Sodium azide (Merck, Darmstadt, Germany) NaN_3, in phosphate-buffered saline was added in a volume of 20 μl to the reaction mixture prior to incubation, to give a final concentration as indicated in the results. In experiments using phenylbutazone this agent (Geigy Basel, Switzerland) was dissolved in phosphate-buffered, gelatinized, heparinized Hank's balanced salt solution and further adjusted to physiological pH with 2.8 per cent sodium bicarbonate. The final concentration of phenylbutazone was 2 mg per ml. The reaction mixtures of leucocytes and bacteria were then made up in this medium and incubations carried out as described above.

Blood was obtained from normal adult persons, from one female and four male patients with chronic granulomatous disease, and from eight related female carriers of this disease. The results of leucocyte function studies in these patients and relatives have been presented previously (14).

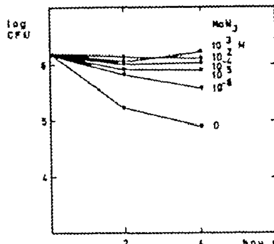


Fig. 1. Effect of increasing molarity of sodium azide (NaN_3) as indicated in the figure, upon the total recoverable colony-forming units (CFU) *Staph. aureus* from a reaction system of normal leucocytes and *Staph. aureus*.
Mean of two experiments.

RESULTS

Figure 1 shows the effect of sodium azide in graded concentrations upon the total number of CFU recoverable with normal leucocytes. Marked inhibition of the killing of *Staph aureus* by normal leucocytes can be detected with 10^{-6} M azide, and killing appears to be completely prevented by 10^{-4} M. Figure 2 shows, however that the number of recoverable intracellular CFU increases progressively with increasing concentration of azide up till 10 M. Above this molarity azide was bactericidal towards the test-organism Figure 3 shows the effect of sodium azide upon normal cells compared to cells from patients and carriers of chronic granulomatous disease (C.G.D.) if total CFU were recorded. Reduction in total CFU is essentially nill with cells from C.G.D. patients and azide, in concentrations above 2×10^{-3} M, renders normal cells at least as inactive as C.G.D. cells. Cells from heterozygous carriers of C.G.D. are functionally intermediate to normal cells and C.G.D. patient cells or azide treated normal cells.

The effect of 10⁻ M azide upon recoverable intracellular CFU from these three

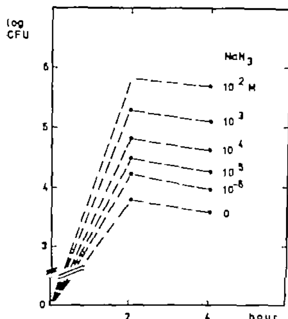


Fig. 2 Effect of increasing molarity of sodium azide as indicated in the figure, upon the intracellular recoverable colony-forming units (CFU) *Staph. aureus* from a reaction system of normal leucocytes and *Staph. aureus*
Mean of two experiments.

groups of cells is shown in Figure 4 There is a marked increase in intracellular CFU when normal cells are treated with azide (circles) A slight increase in intracellular CFU was seen from untreated to azide-treated C.G.D heterozygote cells (triangles) This is not surprising since C.G.D carriers are supposed to harbour a normal and a defective cell population (24) and azide would render all the cells defective. Even if cells from the homozygote C.G.D patients were used, azide seems to increase to a minor extent the number of recoverable intracellular CFU (squares) In Figure 5 the effect of 10^{-4} M sodium azide is compared with that of phenylbutazone 2 mg per ml,—another known inhibitor of intraleucocytic killing (21,23) If total CFU were recorded complete inhibition of killing by normal cells seems to be achieved with both agents, but if intracellular CFU were recorded, the blocking effect of azide seems to be slightly superior to that of phenylbutazone. Since however the effect of azide was slightly decreased in the simultaneous presence of phenylbutazone this might partly be explained by an inhibitory effect of phenylbutazone upon the ingestion, whereby fewer bacteria would be taken up in the cells, and therefore more bacteria killed by the antibiotics in the surrounding medium. Phenylbutazone thus inhibits both ingestion and intraleucocytic killing of *E. coli* by guinea pig polymorphonuclear leucocytes (23)

To study whether azide was bound to the cells normal leucocytes were incubated for 30 minutes at 35 C with rotation in the presence and absence of 2.1×10^{-4} M sodium azide. The cells were then washed thrice at low speed centrifugation followed by reaction with the test-organism in the usual manner The results shown in Figure 6 indicate that azide exerts no permanent effect upon resting cells. This suggests that azide either diffuses freely across the cell mem-

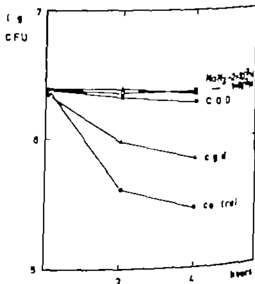


Fig. 3 Total recoverable colony-forming units (CFU) *Staph. aureus* from reaction systems of *Staph. aureus* and 1) normal leucocytes (● ten experiments in four persons) 2) leucocytes from patients with chronic granulomatous disease (C.G.D.) (■ eight experiments in five patients) 3) leucocytes from female carriers of C.G.D. (c g d) (▲ eight experiments in seven carriers) and 4) normal leucocytes in the presence of sodium azide (NaN_3) 2×10^{-4} M (□; four experiments in three persons) and 1×10^{-4} M (△; two experiments in two persons)

It has recently been suggested that superoxide, O_2^- may be formed in human leucocytes during ingestion of particles, and might possess bactericidal activity (3). It is indicated by the present studies showing complete inhibition of intraleucocytic killing by inhibition of myeloperoxidase with sodium azide that if energy release from O_2^- is important for killing this would be via reduction to H_2O_2 and further reaction of H_2O_2 with myeloperoxidase.

The results of the studies shown in Figure 6 indicate that azide exerts no permanent effect on resting cells. One possibility to be mentioned is that the resting cell membrane is not permeable to azide. In that case it would seem likely that azide is taken up along with the ingested particles from the surrounding medium and azide would then be located in critical position in the phagocytic vacuole if myeloperoxidase acts in the vacuole after liberation of granular contents into the vacuole.

The point of importance for the practical application of sodium azide in an in vitro phagocytic system is the finding that this agent effectively blocks intraleucocytic killing of *Staph. aureus* without interfering with the ingestion-phase. A slight stimulation of the ingestion could be indicated by some of the data, but the experiments with azide-treated leucocytes from C.G.D. patients do not support this suggestion. The blocking effect of azide was found to be at least as pronounced as that induced by phenylbutazone which has been used in another modification of the technique aimed at a more precise evaluation of granulocyte function (21). The present studies further illustrate the sensitivity by which defects in intraleucocytic killing can be disclosed by measuring intracellular recoverable CFU illustrating also the superiority of this principle over the isolated recording of total recoverable CFU. Finally since the azide-sensitive antibacterial system seems to be by far the most important of many possible systems for the initial inactivation of a number of micro-organisms, the comparison between azide-treated and untreated cells should be valuable in the evaluation of this system under clinical conditions.

This work was supported by grants from *The Michaelsen Foundation*, *The Danish Medical Research Council*, and *The Foundation for the Advancement of Medical Science*, Copenhagen, Denmark. Mrs. *Ulla Holby* is thanked for excellent technical assistance. *C. Koch* is holder of a research grant from *The Michaelsen Foundation*.

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BRIEF REPORT

ELIMINATION OF ANTIBIOTIC RESISTANCE FACTORS FROM *ESCHERICHIA COLI* EXPOSED TO ANTHELMINTICS

Sigrid Tue Jørgensen

It is well known that antibiotics orally administered to animals will exert a selection pressure on the intestinal micro-organisms towards an antibiotic resistant flora, (*Smith & Crabb* 1957) The determinants of the antibiotic resistance can be located either on the bacterial chromosome or in the cytoplasm on an autonomous piece of DNA. The latter can be eliminated by treatment with several different chemicals and the bacterial host will be cured of its resistance determinants (*Watanabe & Fukasawa* 1961) *Watanabe & Fukasawa* (1961) were the first to grow R factor carrying enterobacteria in broth containing acridine dyes (2,3-5,6 dibenzopyridine derivatives) They did not, however obtain the same high percentage of curing as did *Hirota & Iijima* (1957) with the *E. coli* sex factor F and acriflavine. *Walton* (1972) reports on loss of antibiotic resistance in preliminary experiments with R factor carrying *E. coli* strains grown in the presence of various anthelmintic, anticoccidial and antifluke agents.

The present work was carried out in order to investigate whether widely used anthelmintics would have any curing effect *in vitro* on two strains of *Escherichia coli* carrying multiresistant drug resistance factors. Strain A1B 1 was isolated from faeces from a healthy 3 weeks old calf and shown to carry an R factor mediating resistance to ampicillin, streptomycin, sulphonamides, tetracycline, and chloramphenicol. Strain K 388 was isolated from the intestine of a 4 days old calf that had died from enteritis. K 388 carried an R factor with resistance to the following drugs neomycin, streptomycin, sulphonamides tetracycline, and chloramphenicol. The location of the above mentioned resistance determinants on R factors was established in crosses with *E. coli K 12 F⁻ nal^r* and on elimination by sodium dodecyl sulphate (*Jørgensen* unpublished) An overnight broth culture of each strain was diluted to a density of 10^4 and 10 cells. 0.2 ml of each dilution was inoculated into 2 ml of nutrient broth containing 100 mg/ml of one of the following anthelmintics Septuron Ido vet.® (piperazine-1-thiocarbonic acid) Banminth® (pyrantell tartras NFN) or Thibenzole vet.® (thiabendazolum NFN) After incubation at 38 C on shaker for 6 to 8 hours, 10^n dilutions of each culture were plated on nutrient agar (*Lederberg & Lederberg* 1952) and replicated to a series of Mac Conkey and nutrient agar plates each containing one of the antibiotics to which the R factor initially mediated resistance.

Control cultures of both strains were incubated without anthelmintics to investigate whether spontaneous curing would take place. The results of the attempted curing are shown in Table 1

As the two initial dilutions gave identical results only one appears in the table. Both controls carried all resistance markers, i.e. no spontaneous curing was observed in 400 colonies examined.

From the table it follows that 19 per cent of the examined clones in one strain and 1 per cent in the other had lost all resistance markers during incubation with Septuron Ido vet.® The tetracycline marker alone was lost in 5 per cent of the K 388 clones.

Neither Banminth® nor Thibenzole vet.® were efficient curing agents in these strains, Thibenzole vet.® provoking curing in 2 per cent of the K 388 clones and no R factor elimination at all in A1B 1 Growth in Banminth® containing broth did not induce curing but in two K 388 clones one resistance marker was lost, Thibenzole vet.® and Ban-

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* Septuron Ido vet.® piperazine-1-thiocarbonic acid 30 per cent w/w potato starch 70 per cent w/w Banminth® pyrantel tartrate 12.75 per cent w/w aerosol 0.50 per cent w/w tween-80 (U.S.P.) 0.25 per cent w/w sodium saccharin (NF) 0.40 per cent w/w; sucrose (U.S.P.) 86.10 per cent w/w Thibenzole vet.® thiabendazole 33.3 per cent w/w; antifoam-A 0.37 per cent w/w tween-80 (U.S.P.) 0.02 per cent w/w red iron oxide 0.17 per cent w/w; sucrose (U.S.P.) 66.11 per cent w/w

TABLE 1 *Anthelmintic-induced Loss of R Factors in Two Strains of* E. *coli*

Strain A1B 1

Agent	Frequency of drug-susceptible clones among 100 clones examined					
	neo.	amp.	sm.	su.	tc.	cm.
Septuron Ido vet.®	19	19	19	19	19	19
Banminth®	0	0	0	0	0	0
Thibenzole vet.®	0	0	0	0	0	0

Strain K 388

Agent	Frequency of drug-susceptible clones among 100 clones examined				
	neo.	sm.	su.	tc.	cm.
Septuron Ido vet.®	1	1	1	6	1
Banminth®	1	0	0	1	0
Thibenzole vet.®	2	2	2	2	2

* neo. neomycin amp.: ampicillin sm. streptomycin su. sulphonamides tc. tetracycline cm. chloramphenicol.

minth® were also applied by *Walton* (1972) who was able to eliminate the tetracycline marker in 4 out of 7 examined E. *coli* strains by either of the two agents. The chloramphenicol marker was lost in 3 and 2 strains out of 7 after treatment with Thibenzole vet.® and Banminth® respectively. Unfortunately no detailed information is given of the R factors before the attempted curing so it is not clear whether curing or only partial loss of resistance determinants took place. The number of clones tested for each strain and the percentage of drug susceptible clones among them are not mentioned either. Thus a direct comparison between Walton's work and the present report is not possible, but a closer investigation of the possible role of anthelmintics as curing agents seems justified.

The author is grateful to Dr A. Dam, State Veterinary Serum laboratory Copenhagen who kindly provided strain K 388 and to the firms A/S Ferrosan, A/S Pfizer and Merck Sharp & Dohme as donators of the anthelmintic agents.

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centrifugation at 2,000 × g for 10 min, the supernatant was discarded. The parasites were re suspended in PBS and then spotted on glass microscope slides that had been siliconized in 1 per cent Siliclad (Clay Adams, Parsippany N J) Each dried smear (0.05 ml) contained 25–50 parasites per microscopic field at a magnification of × 320 Very few red and white blood cells were seen. Prepared slides were stored at 4 C.

Preparation of Rabbit Antihuman Gammaglobulin

Rabbits were immunized with 10 mg human gammaglobulin (Cohn Fraction II Kabi, Stockholm, Sweden) emulsified in complete Freund's adjuvant (CFA) (Difco, Detroit, Mich.) via the hind foot pads. After five weeks, the rabbits were injected subcutaneously with 3 mg gammaglobulin emulsified in CFA followed by intravenous (IV) injection 2 weeks later with 5 mg gammaglobulin without CFA. One week later the rabbits were bled from the marginal ear vein.

The gammaglobulin fraction was precipitated by 33 per cent saturation with ammonium sulphate reconstituted in PBS and dialyzed against several changes of PBS. Globulin concentrations were determined by optical density readings at 280 nm using $E^{1\%}_{280\,nm}$ = 15 (20) At a concentration of 5 mg/ml, the antiglobulin before peroxidase labeling contained 8 units of antiglobulin/ml by immunodiffusion against 1 mg/ml human gammaglobulin (6) The antiglobulin gave an immunoelectrophoretic pattern for IgG against human serum.

Preparation of Peroxidase-labelled Antibody

Horseradish peroxidase (Grad VI Sigma, St. Louis, Mo.) was coupled by glutaraldehyde to rabbit antihuman gammaglobulin according to Avrameas (2) 24 mg peroxidase was mixed with 12 mg rabbit antihuman gammaglobulin contained in 2 ml of 0.1 M phosphate buffer pH 6.8 followed by addition of 0 1 ml 1 per cent glutaraldehyde (25 per cent equeous solution, TAAB Reading England) This mixture was stirred t room temp for 3 h and then dialyzed for 24 h against PBS at 4 C. This was followed by centrifugation at 20,000 × g for 20 min at 4 C. The peroxidase-labelled antibody was separated from free peroxidase by gel filtration through a 500 ml Sephadex G-100 column in PBS (Fig 1) The first peak eluted after exclusion of the void volume contained the conjugate and was concentrated by pervaporation at 4 C, then dialyzed against PBS and stored at 4 C The conjugate had a molar ratio of peroxidase to antibody of 2 as determined from standard curves for absorption of peroxidase at $E_{280\,nm}$ and $E_{403\,nm}$ and of antibody at $E_{280\,nm}$.

The optimal dilution of the conjugate used for titration was 1/20 as obtained by checkerboard titrations (9) Before absorption with rat liver powder the conjugate at a 1/20 dil tion contained 0.4 mg/ml protein as determined from a standard curve for absorption at $E_{280\,nm}$.

The conjugate was absorbed with acetone-dried (lyophilized) rat liver powder using 20 mg liver powder/ml conjugate. This mixture was left at room temp for 3 h with occasional mixing followed by centrifugation at 48,000 × g for 1 h at 4 C.

Immunoperoxidase Method

All sera used were first heated at 56° C for 30 min to inactivate complement. Slides containing smears of *Toxoplasma* were rinsed in PBS and distilled water air-dried covered with a dilution of human serum prepared in 2 per cent rabbit serum (NRS) and incubated for 30 min at room temp in a moist chamber This was followed by two 15 min rinses in cold PBS a 2 min rinse in distilled water and air-drying. After addition of peroxidase-labelled rabbit antihuman gammaglobulin, the slides were incubated for 30 min at room temp in a moist chamber followed again by two 15 min rinses in cold PBS and a 2 min rinse in distilled water The smears were stained for enzymatic activity by the method of *Graham & Karnovsky* (13) by incubating at room temp for 20

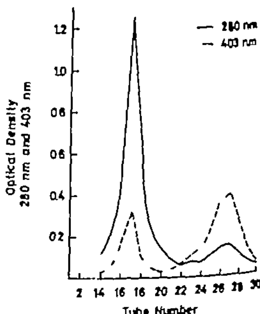


Fig 1 This flow diagram shows gel filtration on Sephadex G-100 of peroxidase-labelled rabbit antihuman gammaglobulin. Approximately ten ml of eluate was collected per tube T bes 14 through 20 contained the conjugate and were pooled.



Fig 2 This photograph shows an IIPT *positive* reaction by *Toxoplasma* × 1000.

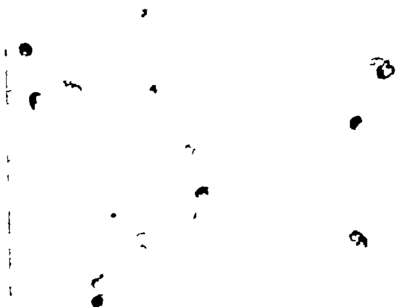


Fig 3 This photograph show an IIPT *negativ* reaction by *T xoplasma* × 850.

of brown or gray color of *Toxoplasma* parasites (Fig. 2 and 3). The titre endpoint could thereby be more easily judged.

No apparent difference in titres was observed between untreated slides and those pretreated with 5 per cent NRS to block non specific binding by antibodies. Also treatment with 2 per cent OsO_4 in distilled H_2O for 2 min to post-oxidize the parasites just before mounting in order to increase the intensity of the brown color was not necessary.

The IIPT for antibodies to *Toxoplasma* was compared with the DT and IHAT in terms of their specificity and sensitivity (Table 1). Identical titres were obtained with the IIPT and DT in 17 out of the 31 sera, and a one titre difference between the two tests was observed in 12 sera. In 2 sera, a 2 titre difference was found between the two tests.

Identical titres were obtained with the IIPT and IHAT in 4 out of the 31 sera, and a one titre difference was found between these two tests in 13 sera (Table 1). In 8 sera a 4 titre difference was found, in 4 sera there was an 8–16 titre difference, and 2 sera had a 32–64 titre difference between the two tests.

DISCUSSION

Excellent titre agreement was found between the IIPT and DT, as identical titres or a one titre difference were found in 29 out of the 31 sera when comparing results of the two tests (Table 1). However, more sera should be tested to further verify the specificity and correlation of the IIPT with the DT.

Excellent titre agreement between the IFAT and DT was reported by *Walton et al.* (25) although the IFAT tended to yield higher titres. In the present investigation, the IIPT also tended to yield higher titres than the DT (Table 1). In addition, the IIPT does not seem to be as time-consuming to perform as the IFAT.

With experience it was not difficult to distinguish between positive and negative reactions in titration experiments. The parasite color of the negative PBS and normal human serum control slides was important in judging endpoint titrations. The reproducibility of the IIPT was also good when titres were determined by different individuals.

Endogenous peroxidase activity by *Toxoplasma* was reported by *Akao* (1) to be confined to mitochondria. In the present investigation, pretreatment of *Toxoplasma* with the methanol HCl solution to inhibit endogenous peroxidase activity had little effect on final endpoint titres when compared with those of untreated parasites. Therefore pretreatment of smears with methanol HCl was considered optional.

The results indicate that the immunoperoxidase method can be successfully used for the serological diagnosis of toxoplasmosis. Advantages of the IIPT are that reagents can be obtained commercially, only a light microscope is needed, the test is not difficult to perform technically, results can be obtained quickly and slides can be stored for reference. The test could therefore be applicable in many different laboratories.

Addendum. After this paper had been accepted for publication the authors have been aware of a paper published by *Am stak et al.* (27).

The authors thank Miss *Wibecke Aasnas* for excellent technical assistance.

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DETECTION OF AUSTRALIA-SH ANTIGEN IN SERUM

A Comparison of the Electron Microscopical Agar Gel Double Diffusion and Complement Fixation Tests

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Presence of Australia-SH antigen in serum has been studied by agar gel double diffusion electron microscopy and complement fixation tests. Serum from 30 patients suffering from serum hepatitis or infectious hepatitis and serum from 10 apparently healthy blood donors have been included in the investigation. In serum from four of the patients with Australia-SH antigen positive serum hepatitis, the presence of the antigen has been studied during the course of the disease. The agar gel double diffusion test was less sensitive than the other two methods, although the sensitivity of this method was raised by increasing the amount of the serum sample to be examined. The present electron microscopical test seems to be at least as sensitive as the complement fixation test. However the electron microscopical test may be less specific than previously believed.

Demonstration of Australia-SH antigen in serum has mainly been performed either by agar gel double diffusion (*Blumberg & Alter* 1965 *Prince* 1968) or by complement fixation (*Shulman & Barker* 1969 *Purcell et al* 1969) tests. Several variants of counterelectrophoretic techniques (*Bedarida et al* 1969 *Pesendorfer et al.* 1970 *Prince & Burke* 1970) have also become commonly used. However the haemagglutination and haemagglutination inhibition (*Vyas & Shulman* 1970) radioimmuno-assay (*Walsh et al.* 1970) platelet aggregation (*Melartin et al.* 1970) immune adherence haemagglutination (*Okochi et al.* 1970) and latex agglutination (*Leach & Ruck* 1971) tests have all been used for detection of the Australia-SH antigen in serum.

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By electron microscopy the Australia-SH antigen has been associated with three morphologically different structures in serum. These are small globular particles of diameter 20–25 nm (*Bayer et al.* 1968 *Prince* 1968) tubular elements of about the same diameter and of varying lengths (*Bayer et al.* 1968) and larger globular particles of diameter 40–46 nm, sometimes with visible substructure (*Dane et al* 1970 *Solaas* 1970 a) (Fig 1) The Australia-SH antigen in serum has previously been studied by electron microscopy after partial purification of the antigen by centrifugation (*Bayer et al.* 1968, *Prince* 1968 *Almeida et al* 1969 *Cossart et al.* 1971) An agar-diffusion filtration procedure has also been employed (*Kelen et al.* 1971)

The Australia-SH antigen has been shown to be strongly associated with serum hepatitis (*Prince* 1968, *Krugman & Giles* 1970, *Solaas et al* 1971) However the antigen has been demonstrated to occur at low frequency in

serum of apparently healthy blood donors (*Prince* 1968 *Gocke et al* 1969 *Blumberg et al.* 1970 *Solaas* 1970 b)

In the present investigation, serum from patients with serum hepatitis or infectious hepatitis and from blood donors has been examined for a presence of the Australia-SH antigen. The serum samples have been studied by a recently developed electron microscopical method (*Solaas 1973*) by agar gel double diffusion and complement fixation. The aim of the study is to compare the results obtained by these three methods.

MATERIALS AND METHODS

Patient and blood donor sera. Sera of all patients and blood donors included in the present investigation have been studied previously (*Solaas* 1970 b *Berg et al.* 1971 *Solaas et al* 1971) The sera were stored at −40° C until the present examination. Where nothing else is stated, only the first serum sample obtained from an individual has been analyzed. Depending on the clinical diagnosis, the persons have been divided into four groups

Serum hepatitis patients (SH) Ten patients out of 13 diagnosed as suffering from serum hepatitis or possible serum hepatitis (*Solaas et al* 1971) were selected on the following criteria *Firstly* four patients who on no occasion during their disease could be shown to harbour Australia-SH antigen in their serum, as judged from agar gel double diffusion experiments, were included. *Secondly* four out of nine patients, all of whom had the antigen in their serum, were selected. All serum samples obtained from these patients were studied. Clinical data are given below *Thirdly* two patients were selected randomly among the remaining five.

Infectious hepatitis patients (IH) Ten patients were selected randomly among 35 who presumably were suffering from infectious hepatitis (*Solaas et al.* 1971)

Epidemic hepatitis patients (EH) Ten patients among 23 who contracted the disease during point source epidemic of hepatitis (*Berg et al* 1971) were randomly selected.

Blood donors (BD) Ten apparently healthy blood donors were included in this group The Australia-SH antigen was known to be present in serum of two of these as revealed by agar gel double diffusion tests (*Solaas* 1970 b)

Antisera to the Australia-SH antigen Serum from a patient who had previously suffered from track-finders hepatitis (*Berg et al* 1971) was used in the electron microscopy experiments. Antibodies to normal serum proteins have not been found in this serum sample (*Solaas 1973*)

Serum E-Y (*Solaas & Berg* 1970) obtained from a multiply transfused patient was employed both in the agar gel double diffusion test and in the complement fixation test. In the latter experiments, serum from another multiply transfused patient (*Solaas et al* 1970) was used as well.

Agar gel double diffusion tests. All serum samples were tested by agar gel double diffusion as previously described (*Solaas & Berg 1970*)

Selection of sera to be included in the present study was based on previous results of agar gel double diffusion tests. The serum samples lacking the Australia-SH antigen as judged from the initial tests, were retested. This time the wells were refilled once and twice after the serum had diffused completely into the agar At the time of the last filling antiserum was applied to the central well on the slide

Some of the serum samples, initially shown to contain the Australia-SH antigen, were titrated by dilution in 0.85 per cent (w/v) NaCl before retesting.

Electron microscopy Electron microscopy was performed as previously described (*Solaas* 1973).

The serum samples were coded by a person who kept the code until the results had been finally scored by the examiner

For each serum sample nine squares evenly distributed throughout a 200 mesh copper grid were examined. The examination lasted for about 30 minutes. The Australia-SH antigen was considered to be present in the samples if one or more of the following phenomena (see Fig 1) were observed

a. Aggregates consisting of small globular Australia-SH antigen associated particles.

b Single tubular Australia-SH antigen associated element.

c Single larger globular Australia-SH antigen associated particle.

d. Aggregate consisting of different Australia-SH antigen associated particles

The total number of observations of the Australia-SH antigen in a sample was recorded.

Complement fixation A microtitre complement fixation test used in the routine in this laboratory was employed. The test is based on the method of *Sever* (1962) and the details are as follows

The sera were incubated at 56 C for 30 min to destroy the complement activity Serum dilutions were performed in barbital buffer of pH 7.2-7.4 Box titration experiments on the antisera and on a control serum containing the Australia-SH antigen have shown that suitable dilutions in the complement fixation test were ½ and 1/ both for the antisera and for the sera to be investigated (unpublished results) A positive and a negative control was always included in the test.

Equal volumes (0.025 ml) of diluted serum

TABLE 1 *Incidence of the Australia-SH Antigen in Serum of Patients and Blood Donors as Judged by Three Methods*

Group of persons	Method								
	Agar gel double diffusion*			Electron microscopy			Complement fixation		
	Australia-SH antigen		Total	Australia-SH antigen		Total	Australia-SH antigen		Total
	present	absent		present	absent		present	absent	
SH	6	4	10	8	2	10	4	3	7‖
IH	1	9	10	3	7	10		8	10
EH	0	10	10	3	7	10	0	10	10
BD	2	8	10	6	4	10	2	8	10
Total	9	31	40	20	20	40	8	29	37

* Serum samples initially found to lack the Australia-SH antigen have been tested after refilling of the wells.
‖ Three samples could not be tested because of scarcity of serum.
SH Serum hepatitis patients IH: Infectious hepatitis patients EH Epidemic hepatitis patients BD Blood donors.

TABLE 2. *Incidence of Australia-SH Antigen in Serum Samples During Australia-SH Antigen Positive Hepatitis of Four Patients Obtained by Three Methods**

Method	Australia-SH antigen		Not tested	Total
	present	absent		
Agar gel double diffusion‖	5	10	0	15
Electron microscopy	9	6	0	15
Complement fixation	10	4	1	15

* The first serum samples obtained from these patients are omitted.
‖ Serum samples initially found to lack the Australia-SH antigen have been tested after refilling of the wells.

drawn and analysed, is included in Table 1 and omitted from Table 2.

As shown in Table 1 agar gel double diffusion analyses revealed a presence of the Australia-SH antigen in 23 per cent of the individuals electron microscopy revealed a presence in 50 per cent and, using the complement fixation test 22 per cent were found to harbour this antigen. Among the serial samples obtained during the course of serum hepatitis in four patients, agar gel double diffusion showed that 33 per cent harboured the antigen using electron microscopy and complement fixation tests 60 per cent and 67 per cent, respectively were found to harbour the antigen.

Agar Gel Double Diffusion Tests

All serum samples which by agar gel double diffusion tests were shown to contain the Australia-SH antigen either initially or following retesting with larger amounts of serum, have been included in Tables 1 and 2.

A doubling of the quantity of serum obtained from the individuals (Table 1) in the test, showed that one patient belonging to the group IH, in whom antigen in serum could not be demonstrated initially did possess the Australia-SH antigen. The antigen in this serum sample was demonstrable also by electron microscopy and complement fixation. The amount of serum from another patient in this group was not suffici-

TABLE 3 *Number of Serum Samples in which Australia-SH Antigen is Demonstrable by Electron Microscopy as Single Particles and/or Aggregates Before and After Addition of Specific Antiserum*

Group of persons	Only single particles		Single particles and aggregates		Only aggregates	
	A	B	A	B	A	B
SH	6	3	4	3	1	8
IH	1	1	0	1	0	0
EH	0	2	0	0	1	0
BD	2	1	1	2	1	0
Total	9	7	5	6	3	8

A: Antiserum not added B Antiserum added SH, IH, EH, BD See Table 1

TABLE 4 *Number of Individuals with Different Reaction Patterns with Respect to Presence and Absence of Australia-SH Antigen as Revealed by Three Methods*

Group of persons	Method	Number with reaction pattern									Total
	AGDD	+	+	+	+	+	–	–	–	–	
	EM	+	+	+	–	–	+	+	–	–	
	CF	+	NT	–	+	–	+	–	+	–	
SH		3	3	0	0	0	1	1	0	2	10
IH		1	0	0	0	0	0	2	1	6	10
EH		0	0	0	0	0	0	3	0	7	10
BD		2	0	0	0	0	0	4	0	4	10
Total		6	3	0	0	0	1	10	1	19	40

AGDD Agar gel double diffusion EM Electron microscopy; CF Complement fixation; + Australia-SH antigen present; – Australia-SH antigen absent NT Not tested SH, IH, EH, BD See Table 1

ent for retesting in agar gel double diffusion experiments.

Among the serum samples obtained from the patients in the group SH during disease (Table 2) refilling of the wells once (for details, see below) showed that four additional samples contained the Australia-SH antigen.

A further increase in the amount of serum to be tested did not reveal any additional serum sample in which the Australia-SH antigen was present.

Electron Microscopical Examinations

For electron microscopy all serum samples and the antiserum were diluted 1/100 and 1/1,000 respectively with 0.85 per cent (w/v) saline, based on the results of a previous investigation (*Soleas* 1973) Four aliquots of each serum sample were examined in the electron microscope the two serum dilutions mentioned and these serum dilutions after addition of equal amounts of antiserum correspondingly diluted with saline.

The features observed in serum samples which by electron microscopy were found to contain the Australia-SH antigen are presented in Table 3 In a total of eight samples, aggregated Australia-SH antigen associated particles were found without addition of antiserum. In six of these samples, tubular and/or larger globular Australia-SH antigen associated elements were seen together with

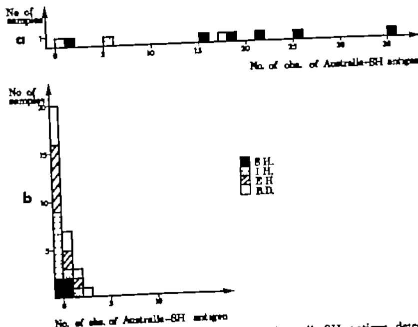


Fig 2 The relation between the number of observations of Australia-SH antigen detected by electron microscopy (see text) and the results obtained by agar gel double diffusion experiments is shown. *a* Serum samples possessing the Australia-SH antigen as judged from the agar gel double diffusion tests. *b* Serum samples without the Australia-SH antigen as judged from the agar gel double diffusion tests. SH, IH EH, BD: see Table 1

smaller globular particles in the aggregates, whereas the remaining two samples contained aggregates consisting of the smaller particles only These two samples were obtained from two of the patients in the SH group who ave been studied during disease Tubular Australia-SH antigen associated elements have been observed in other serum samples from these patients.

Single particles, indistinguishable from the tubular Australia-SH antigen associated structure, were observed in 13 serum samples after addition of antiserum specific for the Australia-SH antigen.

Complement Fixation Tests

Only serum samples which exhibited positive reactions with both antisera employed in the complement fixation tests have been scored as containing the Australia-SH antigen.

Serum obtained from a total of seven persons showed faint anticomplementary activity Anticomplementary activity was not observed in any of the serum samples obtained during the course of serum hepatitis.

Comparison of the Agar Gel Double Diffusion Electron Microscopical and Complement Fixation Tests

Table 4 shows the reaction pattern to be obtained by the three methods with respect to presence or absence of the Australia-SH antigen in persons in each group All sera which by agar gel double diffusion tests were shown to contain the Australia-SH antigen were by electron microscopy found to contain particles associated with the Australia-SH antigen. By the complement fixation test six out of these nine persons were found to possess the Australia-SH antigen in serum too, whereas the remaining three could not be tested by the latter method because of scarcity of serum.

In serum from 31 persons, the Australia-SH antigen could not be demonstrated by the agar gel double diffusion test. However

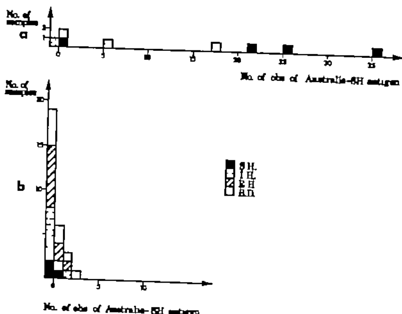


Fig. 3 The relation between the number of observations of Australia-SH antigen detected by electron microscopy (see text) and the results obtained by complement fixation is shown. *a* Serum samples possessing the Australia-SH antigen as judged from the complement fixation tests. *b* Serum samples without the Australia-SH antigen as judged from the complement fixation tests. SH, IH, EH, BD: see Table 1

whether tested by electron microscopy or by complement fixation, one of these persons was shown to possess the antigen in serum. Furthermore, serum from 11 individuals was only by one of the employed methods found to contain the Australia-SH antigen. In 10 subjects, or 25 per cent of all the individuals, the antigen was observed by electron microscopy only. These persons were found throughout all four groups. By complement fixation alone the Australia-SH antigen was demonstrated in serum of one patient who had been assigned to the IH group.

The Australia-SH antigen was by all three methods found to be absent in serum of 19 individuals.

Number of Observations of Australia-SH Antigen Associated Structures Using Electron Microscopy Related to the Results Obtained by Agar Gel Double Diffusion Tests

In this and the next paragraphs, the number of observations of Australia-SH antigen by electron microscopical examinations represents the total count in the four aliquots of one serum sample, based on the criteria given in Materials and Methods.

Fig. 2 shows the reaction pattern of serum from each individual against antiserum to the Australia-SH antigen, using agar gel double diffusion tests, compared to the number of observations of Australia-SH antigen associated structures by electron microscopy. The number of observations of Australia-SH antigen in the samples which by agar gel double diffusion were shown to contain the antigen ranges between 1 and 36 (Fig. 2 a). In two of these nine samples, one in the SH group and one in the BD group observations of the Australia-SH antigen, using electron microscopy amounted to three or less.

By electron microscopical examination, the number of observations of the Australia-SH antigen was three or less in all of 11 serum samples which did not contain the antigen as revealed by agar gel double diffusion (Fig. 2 b). The Australia-SH antigen was absent in 20 individuals as judged both by agar gel double diffusion and electron microscopy.

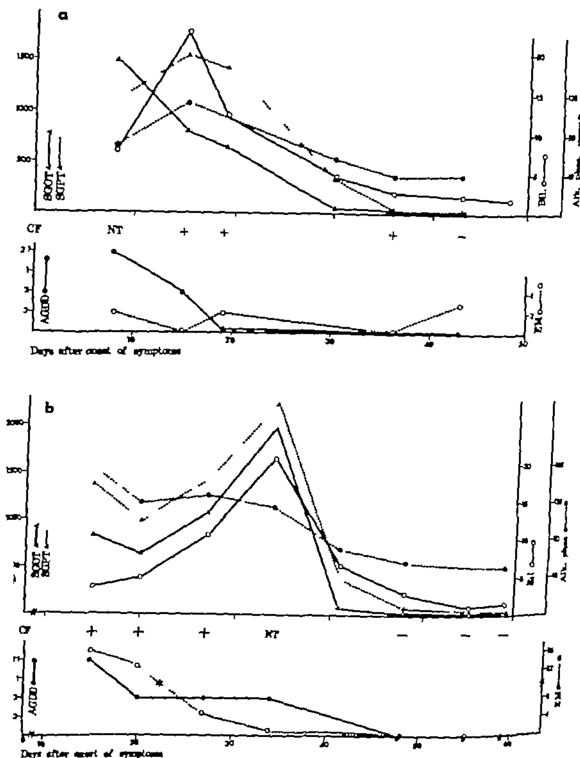


Fig 4 Liver function and Australia-SH antigen data on four patients during the course of serum hepatitis. Australia-SH antigen in serum has been measured by agar gel double diffusion lectron microscopy and complement fixation tests. *a* Case 1 *b* Case 2. *c* Case 3 *d* Case 4 SGOT Serum glutamic oxalo-acetic transaminase SGPT: Serum glutamic pyruvic transaminase Bil. Bilirubin Alk. phos. Alkaline phosphatase AGDD Agar gel double diffusion titration (2 1 2:2 2:3 indicates volumes of serum

c

SGOT
SGPT

Bil.
Alk. phos.

CF NT + + +

AGDD

EM

Days after onset of symptoms

d

SGOT
SGPT

Bil.
Alk. phos.

CF + + +

AGDD

EM

Days after onset of symptoms

volumes of saline 1, 2, and 3 means wells filled once, twice and thrice respectively)); EM Electron microscopy number of observations of the Australia-SH antigen; CF Complement fixation; + Australia-SH antigen present; – Australia-SH antigen absent; NT not tested.

Number of Observations of Australia-SH Antigen Associated Particles Using Electron Microscopy Related to the Results of Complement Fixation Tests

In Fig 3 the number of observations of the Australia-SH antigen by electron microscopy in serum of each of the individuals is compared with the results of the complement fixation tests. Out of eight samples, all containing the Australia-SH antigen as judged from the complement fixation experiments, electron microscopy revealed that one in the group IH did not possess the antigen. In two additional samples, one in the SH group and one in the BD group only one observation of Australia-SH antigen associated structures was made by the latter technique (Fig 3 a)

Ten serum samples which according to the results of the complement fixation tests, did not contain the Australia-SH antigen, were found to contain Australia-SH antigen associated structures. However at most three observations were made in any individual sample (Fig 3 b)

Nineteen serum sample were negative with both methods.

Australia-SH Antigen During Acute Hepatitis

To evaluate the efficiency of the methods by which to detect the Australia-SH antigen during the course of hepatitis four patients the SH group were selected for serial .ts. They had all had Australia-SH antigen in serum as judged from previous agar gel double diffusion experiments (the well filled only once) Furthermore, in all cases the antigen had disappeared in the last sample obtained as judged from the same test.

In one case it was a male aged 25 who developed hepatitis with fatigue anorexia, nausea and scleral jaundice four months after an inoculation against smallpox. The results of the liver function tests are shown in Fig 4 a.

The other three cases were females who were 60 52, and 50 years old at the time of the illness. They all developed jaundice about 14 days after onset of symptoms which in all instances were malaise and nausea. Discolouration of stools and urine was observed in all. A diagnosis of "possible serum hepatitis" was established because they had all undergone procedures such as surgical incisions, dental treatment, injections, or inoculations within the last six months before onset of disease (*Solaas et al* 1971) The results of the laboratory tests of the liver function in these patients are shown in Fig. 4 b, c, and d respectively

A total of 19 serum samples from these four patients have been examined. In Fig. 4 the results of the Australia-SH antigen determinations by agar gel double diffusion titration, electron microscopy and complement fixation are presented together with data on the liver function in the four patients. In all instances the peak of the Australia-SH antigen content in serum of the patients, as measured by agar gel double diffusion titration experiments and electron microscopy seems to have occurred before the peak values from the liver function tests. However small amounts of antigen were present in serum of three of the patients (Fig 4a, c, and d) at the end of the observation period, whether measured by complement fixation or by electron microscopy

DISCUSSION

Demonstration of the Australia-SH antigen by electron microscopy is based on visual recognition of the three morphologically different structures previously found to be associated with this antigen (*Prince* 1968, *Bayer et al.* 1968 *Dane et al* 1970) Antiserum specific to the Australia-SH antigen agglutinates these structures. The electron microscopical test employed in this study has been found suitable to detect the Australia-SH antigen (*Solaas 1973*)

Serum samples with a presumed presence of Australia-SH antigen particles, revealed by the electron microscopical test were more numerous than those which by agar gel double diffusion or by complement fixation

Number of Observations of Australia-SH Antigen Associated Particles Using Electron Microscopy Related to the Results of Complement Fixation Tests

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Serum samples with a presumed presence of Australia-SH antigen particles, revealed by the electron microscopical test were more numerous than those which by agar gel double diffusion or by complement fixation

tests were found to contain the antigen. Among the serum samples obtained during the course of serum hepatitis, the number of Australia-SH antigen-containing samples found by electron microscopy was about the same as that found by the complement fixation test. Thus, with a view to revealing this antigen, the former test is at least as sensitive as the complement fixation test, and more sensitive than the agar gel double diffusion test, although the sensitivity of the latter test could be increased by increasing the amount of the serum sample to be tested.

These results are in agreement with those obtained by *Cossart et al.* (1971). Likewise *Droukas et al.* (1972) found electron microscopy to be somewhat more sensitive than the agar gel double diffusion test. In a study of different dilutions of Australia-SH antigen-containing sera *Shulman & Barker* (1969) found an increasing sensitivity of the three test systems in the following order: agar gel double diffusion, electron microscopy and complement fixation.

However, the electron microscopical test seems to be less specific than previously presumed. Structures indistinguishable from the Australia SH antigen associated particles have been observed by electron microscopy in serum of an apparently healthy student (*Solaas* 1973). In the present study small amounts (less than four observations per sample) of presumed Australia-SH antigen could be identified using this test as the only method, in 33 per cent of the patients from a point source epidemic of hepatitis, and in 50 per cent (four out of eight) of the healthy blood donors.

At face value this could imply that small quantities of Australia-SH antigen are present in a considerable number of healthy persons. In agreement with this *Apostolov et al.* (1971) found by counter electrophoresis and complement fixation, but not by agar gel double diffusion, a presence of Australia-SH antigen in urine concentrates obtained from two out of seven healthy persons. The antigen was not demonstrable in their sera. Furthermore, it has been calculated that only about 25 per cent of serum hepatitis cases may be prevented by exclusion of blood donors whose sera react with antiserum to Australia-SH antigen, as determined by agar gel double diffusion tests (*Gocke et al.* 1970).

On the other hand, if sera where three or less observations were made contain structures which are not true Australia SH antigen particles, this diagnostic procedure failed in four out of 14 serum samples (28.6 per cent) which by agar gel double diffusion were shown to possess the Australia-SH antigen and in seven out of 18 (28.9 per cent) samples where the antigen was detected by the complement fixation test.

In the present study addition of antiserum specific to the Australia-SH antigen prior to electron microscopical examination failed to aggregate the particles observed in seven serum samples. This suggests that some of the structures observed are not true Australia SH antigen particles, unless the immunological test system was greatly out of balance (*Solaas* 1973). Whether the particles observed in the healthy blood donors were agglutinated by the antiserum could not be evaluated. The antigen in three samples was observed only if no antiserum had been added and in one sample only after addition of antiserum.

In eight samples aggregated presumed Australia-SH antigen associated particles were observed by electron microscopy prior to addition of antiserum. None of these sera showed anticomplementary activity. Furthermore, antibody to the Australia-SH antigen could not be observed in any of these samples, either by agar gel double diffusion or by complement fixation tests (unpublished results). The significance of a presence of such aggregates in serum samples is not known.

The use of an antiserum specific to the Australia SH antigen and a presence of proper controls ensure the specificity of the agar gel double diffusion test.

Antisera obtained from multiply transfused patients may contain more than one antibody specificity and antigen-antibody reactions other than the specific Australia-SH reaction

may take place in the complement fixation test. In the present investigation two different antisera were employed. Only serum samples which showed a positive reaction with both antisera have been scored as containing the Australia-SH antigen.

In the present study the agar gel double diffusion and complement fixation tests appeared to be highly specific whereas this was not the case with the electron microscopical test. The real nature of the structures observed in the samples which were not shown by either of the other two methods to contain the Australia-SH antigen is not known. Human serum β lipoprotein resembles in size and appearance closely the smaller 20–25 nm, particles associated with the Australia SH antigen (*Tornvik et al* 1970) and it is conceivable that normal serum may also contain unidentified particles which are morphologically indistinguishable from the tubular and larger globular particles.

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TYPING OF *HAEMOPHILUS INFLUENZAE* BY COUNTERIMMUNOELECTROPHORESIS

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One hundred and eighty-four *Haemophilus influenzae* isolates typed by gel precipitation technique were cultivated in Levinthal broth and the cultures studied by counterimmuno-electrophoresis. All 57 typable strains formed demonstrable type-specific capsular antigen. No cross reaction between the types occurred under the test conditions used. None of the non-typable isolates produced detectable antigen.

Encapsulated strains of *H. influenzae* may be divided into six serological types, designated a–f. The type-specific capsular material has been identified as polysaccharide and diagnostic antisera without crossreactivity can be produced (1). Typing of isolates is traditionally carried out by agglutination or capsular swelling tests. Other methods such as gel precipitation procedure and immunofluorescence technique have been described (7, 9).

Counterimmunoelectrophoresis (CIE) is a new sensitive immunoprecipitation method h[as] been employed successfully for detection of specific antigen present in body fluids during severe infections (3, 4). Encapsulated strains grown in liquid culture will normally release type-specific capsular antigen. The present paper describes the detection of such antigen by CIE and demonstrates the suitability of this typing procedure.

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MATERIAL AND METHODS

Bacterial Strains

Reference strains. The following reference strains were used: A, B_1, C_1, D_1, E-Montenegro and F-Dingles (5).

Clinical isolates. All other strains were isolated during ordinary routine from clinical cases and preserved by freezedrying. Gram negative, non-haemolytic organisms, showing satellitism on blood agar with Staphylococcus streak, were tested for iridescence on Levinthal agar. Only non-motile isolates requiring both X and V factor were termed *H. influenzae* and typed by gel precipitation. Motility was judged by microscopy of liquid culture and growth factor dependence tested on Brain Heart agar with streaks of *Sarcina lutea* and *Streptococcus faecalis* (8).

Media and Cultivation

Blood agar and chocolate agar were prepared from horse blood. Brain Heart Infusion agar (Difco) was used unmodified. Both Levinthal agar and broth were prepared according to *Turk & May* (10).

Cultivation was performed always at 37° C.

Reference Antigen

Type antigen was prepared from all reference strains according to *Omland* (6).

Typing Sera

Rabbit sera were obtained after intravenous immunization with five reference strains (5). A multivalent serum was prepared by mixing equal amounts of all six type sera.

Preparation of Material for the Test

Gel precipitation. The organisms were grown on chocolate agar for 16 hours and harvested in 1 ml distilled water. The suspension received ultrasonic treatment for one minute.

CIE. Five ml of Levinthal broth was inoculated with colonies from agar plates. After 16 hours incubation the culture was centrifuged and the supernatant fluid tested.

In the case of test performed with multivalent serum it was found advantageous to dilute the test material 1 10.

Rate of Release of Capsular Antigen into Liquid Medium

Six unselected type b isolates were tested as follows. Organisms grown for 16 hours in Levinthal broth were washed twice in broth and the optical density adjusted to 0.15 (Beckman colorimeter model C, wave length 525 mμ). Fresh Levinthal broth (7 ml in glass tubes) was inoculated with 0.2 ml of the suspension (viable count approximately 6 × 10 organisms). Samples were taken after 3, 6, 12 and 16 hours incubation. After centrifugation, the concentration of soluble capsular antigen was estimated by testing twofold serial dilutions of the supernatant fluid by CIE. The highest dilution yielding a visible precipitate was regarded as the titre.

Immunological Procedure

Gel precipitation. Glass slides (26 × 76 mm) were covered with 2 ml 1 per cent agar in physiological saline and wells of 3 mm diameter were cut 5 mm apart. Each slide had two identical hexagonal patterns with one central and six peripheral wells. The central wells received ultrasonically treated organisms and the peripheral wells type serum and reference antigen in alternation. This arrangement offered a reference system. The slides were incubated in a moist atmosphere at room temperature and read after 24 and 48 hours. Type-specific lines could be detected by fusion with a reference line.

CIE. Slides as described were covered with 2 ml 1 per cent agarose in barbitone-barbitone sodium buffer (pH 8.6, ionic strength 0.05). Wells of 3 mm diameter were cut 5 mm apart. Each slide had six pairs in two rows. The cathodic wells were filled with type antisera a-f and the opposing wells received test material. All wells were filled once to the brim. Electrophoresis was performed at room temperature for one hour using constant voltage of 3 V per cm agarose. The slides were inspected immediately for precipitates with oblique lighting against dark background. Identity with known reference antigen was tested in a three-well system with paired antigen wells (2).

RESULTS

A preliminary series of examinations included all reference strains. CIE of 16 hours broth cultures revealed type specific capsular antigens reacting only with homologous type sera. Further study included 148 *H. influenzae* isolates typed by gel precipitation technique. All typable strains grown for 16 hours in Levinthal broth produced demonstrable quantities of specific capsular antigen. The distribution of types is shown in Table 1. Single precipitation lines developed fully after one hour of electrophoresis. In all typable strains, identity with known reference antigen was demonstrated. None of the cultures of non-typable isolates gave precipitation lines. All isolates were tested with a multivalent serum with activity against all six types. All strains typable by conventional technique were detected. Encapsulated isolates were also studied after as much as 48 and 72 hours' growth in Levinthal broth. In some instances such cultures gave precipitation with two or more of the antisera. Further experiments showed that non-capsulated strains could behave similarly. Such precipitates, which were assumed to be related to *other than capsular antigens*, were never found after 16 hours incubation.

TABLE 1. *Typing of* H. influenzae *by Counter Immunoelectrophoresis (CIE) and Gel Precipitation*

Type	Type determination by gel precipitation	Type determination by CIE
a	1	1
b	46	46
c	0	0
d	2	2
e	2	
f	6	6
Non-typable	91	91

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TYPING OF *HAEMOPHILUS INFLUENZAE* BY COUNTERIMMUNOELECTROPHORESIS

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One hundred and eighty-four Haemophilus influenzae isolates typed by gel precipitation technique were cultivated in Levinthal broth and the cultures studied by counterimmunoelectrophoresis. All 57 typable strains formed demonstrable type-specific capsular antigen. No cross reaction between the types occurred under the test conditions used. None of the non-typable isolates produced detectable antigen.

Encapsulated strains of *H influenzae* may be divided into six serological types designated a f The type-specific capsular material has been identified as polysaccharide and diagnostic antisera without crossreactivity can be produced (1) Typing of isolates is traditionally carried out by agglutination or capsular swelling tests. Other methods such as gel precipitation procedure and immunofluorescence technique have been described (7 9)

Counterimmunoelectrophoresis (CIE) a new sensitive immunoprecipitation method has been employed successfully for detection of specific antigen present in body fluids during severe infections (3 4) Encapsulated strains grown in liquid culture will normally release type-specific capsular antigen The present paper describes the detection of such antigen by CIE and demonstrates the suitability of this typing procedure

MATERIAL AND METHODS

Bacterial Strains

Reference strains. The following reference strains were used A B_1 C D_1 E-Montenegro and F Dingles (5)

Clinical isolates All other strains were isolated during ordinary routine from clinical cases and preserved by freezedrying Gram negative, non-haemolytic organisms, showing satellitism on blood agar with Staphylococcus streak, were tested for iridescence on Levinthal agar Only non-motile isolates, requiring both X and V factor were termed *H influenzae* and typed by gel precipitation Motility was judged by microscopy of liquid culture and growth factor dependence tested on Brain Heart agar with streaks of *Sarcina lutea* and *Streptococcus faecalis* (8)

Media and Cultivation

Blood agar and chocolate agar were prepared from horse blood. Brain Heart Infusion agar (Difco) was used unmodified. Both Levinthal agar and broth were prepared according to *Tast & May* (10)

Cultivation was performed always at 37° C.

Reference Antigen

Type antigen was prepared from all reference strains according to *Omland* (6)

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ULTRASTRUCTURAL STUDY OF EARLY STAGES OF ASEXUAL MULTIPLICATION AND MICROGAMETOGONY OF *TOXOPLASMA GONDII* IN THE SMALL INTESTINE OF THE CAT

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Toxoplasma gondii undergoes schizogony and microgametogony in the epithelial cells of the cat ileum. On the basis of cytoplasmic structures, it is impossible to distinguish between the early stages of these processes but nuclear differentiation appears to have occurred. In the early schizont the chromatin is distributed in small patches throughout the nucleus but in the nucleus of the microgametocyte the chromatin is condensed into a few large areas. In the schizont, the first distinguishing cytoplasmic features are the appearance of the dome-shaped membranes of the merozoite anlagen. In the schizogonic process, nuclear division precedes merozoite formation which occurs by multiple internal budding in a manner similar to endodyogeny The appearance of flagella is the first cytoplasmic feature to distinguish the microgametocyte. Microgamete formation is similar to that reported for other coccidian species. The microgamete consists of a dense elongate nucleus anterior to which is a single mitochondrion. The anterior consists of a perforatorium and two flagella between which 4 microtubules run longitudinally

Hutchison et al. (1969 1970) reported the discovery of the coccidian life cycle of *Toxoplasma gondii* in the small intestine of cats. This was confirmed by *Sheffield & Melton* (1970) *Frenkel et al.* (1970) *Piekarski & Witte* (1970) and *Overdulve* (1970) Until this time only the proliferative forms (endozoites) and the cystic forms (cystozoites) occurring in other tissues were known. The method of multiplication employed by these forms was thought to be binary fission before *Goldman et al* (1958) using silver stains and the light microscope, described a process which they termed endodyogeny These findings were subsequently confirmed by the electron microscopic studies of *Ludvik* (1958) *Gavin et al* (1962) *Wanko et al.* (1962) *Garnham et al* (1962) *Olisa* (1963) *Ogino & Yoneda* (1966) *Wildführ* (1964) *van der Zypen & Piekarski* (1967) *Sénaud* (1967) and *Sheffield & Melton* (1968) *Jacobs* (1967) reviewing the literature proposed that endozoites multiply only by endodyogeny *Vivier* (1970) has proposed

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that another process which he termed endopolygeny may occur

Electron microscope studies of the asexual multiplication of the coccidian stages in the small intestine of the cat by *Sheffield* (1970) *Colley* & *Zaman* (1970) and *Piekarski et al.* (1971) revealed a process of multiple internal budding *Piekarski et al.* (1971) also use the term endopolygeny to describe this process

The development of the microgametocyte has been described by *Colley* & *Zaman* (1970) and *Pelster* & *Piekarski* (1971) as similar to that of the Coccidia.

This paper describes and compares the early stages of asexual multiplication and microgametogony occurring at the time of oocyst production in the small intestine of the cat.

MATERIALS AND METHODS

Specific Pathogen Free (SPF) cats were obtained and treated in a manner similar to that described by *Hutchison et al.* (1971) All the cats were examined serologically prior to the experiment and were Dye Test negative but had become Dye Test positive by the time of autopsy

These cats were infected by feeding aseptic mouse brains containing the cystozoites of Strain SSI/119 of *Toxoplasma gondii* and autopsied seven days later The small intestine was immediately removed and divided into portions. A part of each portion was prepared for histological examination, as described by *Hutchison et al.* (1971) to determine the areas suitable for an electron microscope study The adjacent part of each portion was divided into small cubes and fixed in 5 per cent glutaraldehyde in phosphate buffer pH 7.2 for a minimum of 4 hours at 4 C. The tissue was then washed in phosphate buffer for 12 hours and postfixed for 2 hours at room temperature in 1 per cent solution of osmium tetroxide dissolved in the phosphate buffer The tissue was then dehydrated through a series of ethanol and 2 changes of propylene oxide and embedded an Araldite. Thin sections were cut in the LKB microtome stained with uranyl acetate and lead citrate, and examined in an AEI EM6B electron microscope at 50 or 60 Kv

It is appropriate at this stage to list the definitions and terminology used throughout The terminology is essentially as proposed by *Hoare* (1972) for the developmental stages of *Toxoplasma* and by *Scholtyseck* & *Mehlhorn* (1970) and *Scholtyseck et al* (1970) for the ultrastructural aspects The definitions are as follows

Endodyogeny is a process of internal budding resulting in the formation of two daughter organisms within the original parasite. The membranes and anterior organelles of the daughters start to form before nuclear division is complete.

Schizogony in its normal coccidian sense, is a process of multiple nuclear division followed by migration of the nuclei to the periphery of the schizont. Merozoite formation is initiated at this position below the enclosing membrane of the schizont.

Endopolygeny appears to be a process combining both schizogony and endodyogeny Initially nuclear division takes place without formation of daughter organisms. Subsequently formation of the merozoites occurs within the cytoplasm in a manner similar to endodyogeny

RESULTS

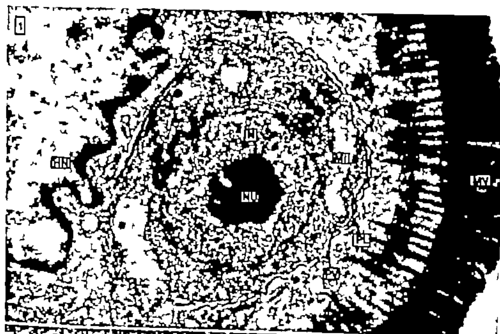
The merozoite enters an epithelial cell of the cat ileum and settles between the host cell nucleus and brush border It becomes a trophozoite and lies within a parasitophorous vacuole This vacuole is bounded by a relatively thick membrane (about 250 Å) which separates the parasite from the host cytoplasm (Fig 1) The infecting merozoite has an ultrastructure comprising of a conoid, rhoptries, micronemes, nucleus with nucleolus, and mitochondria etc. but there is an increase in the number and size of the mitochondria, and the micronemes quickly degenerate as do the rhoptries except for the base of their ducts (Fig 2) At this stage the growing organism becomes more ellipsoidal a change coincident with and perhaps

Fig 1 Transverse section of trophozoite within a parasitophorous vacuole lying between the microvilli and the nucleus of the host cell. × 18 000

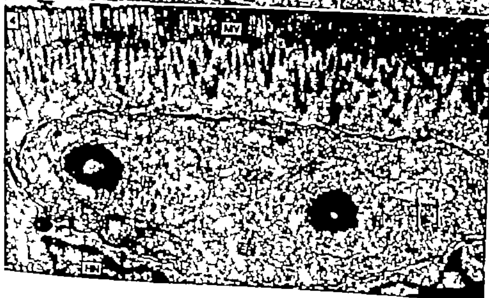
Fig 2 Longitudinal section of a trophozoite displaying conoid ducts of the rhoptries, and a few micronemes. × 19 000

Fig 3 Nucleus of an organism showing the dense body protruding from it. × 14 000

Fig 4 Longitudinal section of binucleate organism displaying the two nuclei each with a nucleolus. Note the finger like protrusion from one of the nuclei (arrowed) × 14 000


1
NU


3
DB


4
MV

caused by the disappearance of the subpellicular microtubules.

Nuclear division gives rise to a binucleate organism (Fig 4) The finger like extrusion of the nucleus observed in Fig 4 has been observed several times but we are uncertain of the significance of this feature. A body similar in size and density to the nucleolus is observed protruding from the nucleus (Fig. 3) This is similar to the structure termed the E-body by *van der Zypen & Piekarski* (1967) Nuclear division may be repeated up to four times. At this stage two types of multinucleate organisms can be distinguished on the basis of changes in their nuclear structure. One type has the chromatin distributed as small areas throughout the nucleus and at the periphery (Fig 5 & 6) At present, we are of the opinion that this is an early schizont. The other type has the chromatin forming large dense areas towards the periphery while the rest of the nucleus contains small dense granules (Fig 7 & 8) and this we consider to be the early stage of the microgametocyte. At this time the cytoplasm of both types of organisms comprises a number of mitochondria golgi bodies, a few polysaccharide and lipid granules, and large amounts of rough endoplasmic reticulum

The earliest stage at which cytoplasmic changes definitely characterise an organism as a schizont is when dome-shaped membranes appear close to each nucleus above the golgi body (Fig 9) and at this stage the nuclei are similar to those described above for the early schizont of Fig. 5 These dome shaped membranes which ultimately become the inner membrane of the merozoites, become associated with a nucleus in pairs (Fig 11) In Fig 10 a single membrane appears to be associated with a nucleus. There are two possible explanations for this firstly it is a diagonal section through one membrane of a pair secondly one nucleus can be associated with a single membrane. We mention the second possibility because of the excessively high proportion of single membranes in the sections which we have examined However this question will only be resolved when we have serial sections through the surrounding cytoplasm of such a nucleus. As each membrane grows posteriorly the 22 subpellicular microtubules appear beneath it and are equally spaced round the periphery Subsequently the conoid and the dense subspherical precursor of the rhoptries arise *de novo* within the membrane. At the base of the dome-shaped membranes, a thickening is evident which appears to be the site of membrane synthesis. A dense structure representing a pole of the nucleus is directed from it towards the opening of the dome shaped membrane (Fig 10) or membranes (Fig. 11) As the dome-shaped membrane elongates the nucleus takes on a horseshoe appearance as it becomes enclosed by the elongation of the dome-shaped membrane or membranes (Fig 12) This elongation of the membrane continues (Fig 13) and finally completely encloses a unit of cytoplasm containing all the organelles found in the merozoite. The daughters grow till they assimilate the cytoplasm of the schizont and completely fill it (Fig 14) Up till this point the schizont has preserved its double membraned pellicle but as the daughters come in contact with the inner membrane it breaks down so that the daughter's membrane lies adjacent to the outer membrane of the schizont (Fig. 15) The outer membrane of the schizont's pellicle now invaginates around the developing merozoites to form the outer membrane of the pellicle of each merozoite (Fig 16) The fully formed merozoites then separate and lie free in the parasitophorous vacuole (Fig 17) They are now mature and ready to leave the host cell and infect new cells

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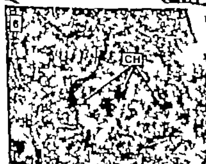
Fig 5 Multinucleate organism showing nuclei characteristic of an early schizont × 18 000

Fig 6 Enlargement of a nucleus from Fig. 5 showing the distribution of chromatin × 25 000

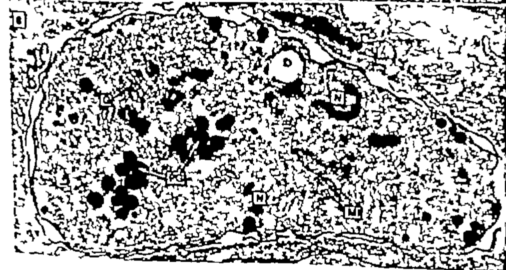
Fig 7 Enlargement of a nucleus from Fig. 8 showing the distribution of chromatin × 45,000

Fig 8 Multinucleate organism showing nuclei characteristic of an early microgametocyte 15 000


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PV

The stage at which cytoplasmic changes definitely characterise an organism as a microgametocyte is when the flagellar structures appear (Fig 18) Before this and before the differences in *nuclear structures already mentioned* are evident the developing microgametocyte cannot be distinguished from the early schizont. When the nuclear differences become apparent the two developmental stages of *Toxoplasma* can be differentiated even in the absence of the more characteristic cytoplasmic structures. As development of the microgametocyte proceeds, the chromatin concentrates at the side of the nucleus near the pellicle while the rest of the nucleus contains small dense granules. The centrioles, which are observed between the nuclei and the periphery develop into the basal bodies and from the latter the flagella arise. The basal bodies are found below the pellicle and *the flagella grow into the parasitophorous* vacuole (Fig 25) A mitochondrion lies close to each nucleus and remains in this position during further development. The dense area of the nucleus and associated *mitochondrion protrudes from the microgametocyte remaining covered by the outer membrane of the* microgametocyte's pellicle, the inner membrane having degenerated (Fig 19) The fate of the light area *of the nucleus* has not been observed. The protrusion of the fully formed microgamete (nucleus, mitochondrion and flagella) continues into the parasitophorous vacuole and there is a thickening of the pellicle *at the base of this protrusion (Fig* [illegible]0) The microgametes separate from the gametocyte and lie free in the parasitophorous vacuole (Fig 21)

The microgamete has a dense elongate nucleus. *Anterior to and partially surrounded* by the nucleus is a single mitochondrion with cristae which are irregularly arranged (Fig 23 & 24) The anterior of the microgamete comprises of an osmiophilic plate forming *the perforatorium* (Fig 24) posterior to *which are the two flagella (Fig 2*[illegible]*) Between* them a row of 4 microtubules run longitudinally for a short distance (Fig. 22 & 23) In certain sections of the anterior there appears *to be an osmiophilic* layer beneath the unit membrane which encloses *the microgamete* (Fig 22 & 23) It is possible that this is all

Fig 9 Early schizont showing the dome-shaped inner membrane of the merozoites. × 15 000.

Fig 10 Portion of a schizont showing the pole of a nucleus directed towards a single developing merozoite × [illegible]6,000

Fig 11 Portion of a schizont *showing a nucleus* becoming associated *with two developing merozoites.* × 24,000

Fig 12 Portion of a schizont showing the nuclei becoming enclosed within the merozoites membrane. Note the thickening at the base of the merozoite membrane (arrowed) × [illegible]5 000

Fig 13 Portion of schizont showing the nucleus almost enclosed by the merozoites membrane Note the thickening at the base of the merozoite membrane (arrowed) × 14,000

Fig 14 Late schizont showing the merozoites fully formed within the pellicle of the schizont. × 12 000

Fig 15 Portion of a schizont showing the merozoite associated with the outer membrane of the schizont to form the merozoites pellicle. Note that between the merozoites the pellicle of the schizont is intact (arrowed) × *21,000*

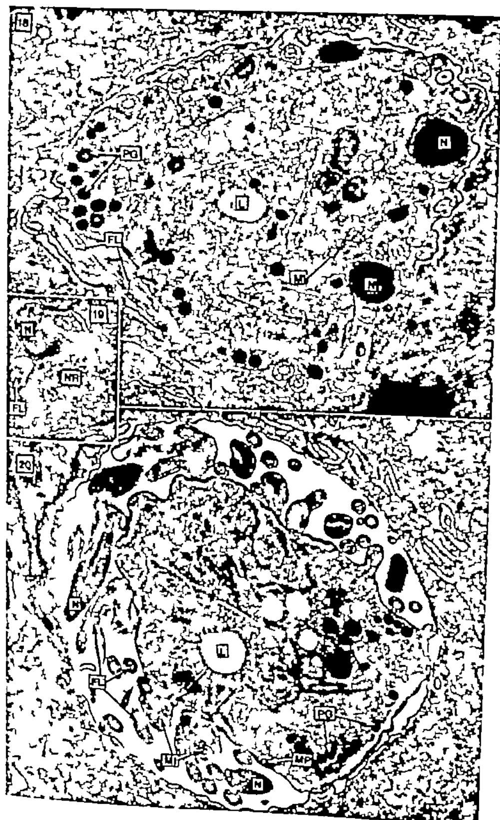
Fig 16 Portion of a schizont showing the intact schizont's pellicle (arrowed) Note the invagination of the outer membrane of the schizont pellicle *to form* the outer membrane of the merozoites pellicle × 15,000.

Fig 17 Shows the mature merozoites lying within the parasitophorous vacuole × [illegible]0,000.

Fig 18 Early microgametocyte *showing* the dense nuclei at the periphery *and the presence* of flagella × 15,000

Fig. 19 Nucleus of microgametocyte *showing the* dense portion extruding into the parasitophorous vacuole and the transparent area towards the interior × 14 000

Fig 20 Late *microgametocyte* showing microgametes *extruding into the parasitophorous* vacuole Note *the thickening at the* base of the extrusion (arrowed) × 13 000

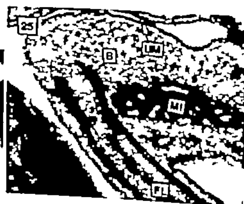


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of our findings, it would appear that initiation of daughter formation is independent of the number of nuclei present. The dense body seen protruding from certain nuclei appears similar to the E-body of *van der Zypen & Piekarski* (1967). This body was said to initiate nuclear division and daughter formation in the endozoites of *Toxoplasma* but its significance in the intestinal forms cannot be decided from our study.

There are a number of ways in which the asexual division which we have studied in the cat intestinal epithelial cells differs at the ultrastructural level, from the "normal" schizogony of *Eimeria* and *Isospora spp.* which have been reported by *Sheffield & Hammond* (1967), *Colley* (1968), *McLaren* (1969), *Snigirevskaya* (1969b), *Schmidt et al.* (1967) and *Lee & Millard* (1971). These could be listed as follows:

1. There is no apparent movement of the nuclei to the periphery.
2. The pellicle remains intact as two membranes and does not lose the inner membrane at an early stage but only when the internally formed daughters come in contact with it.
3. Daughter formation begins within the cytoplasm and not at a point directly beneath the enclosing membrane.
4. Daughters develop internally and not by protrusion from the surface of the schizont.

Both processes are similar in that the outer membrane of the schizont's pellicle gives rise to the outer membrane of the daughter pellicle.

The process which we have studied differs from endodyogeny in the following:

1. Some nuclear division occurs before the initiation of daughter formation.
2. More than two daughters are formed within the parent organism.

Both processes are similar in that the inner membrane of the pellicle degenerates when the daughters come in contact with it and the outer membrane of the parent organism gives rise to the outer membrane of the daughters.

Asexual multiplication in the endogenous forms of *Toxoplasma* is similar to the process described by *Roberts et al.* (1970) and *Sampson & Hammond* (1972) for *Eimeria callospermophili* and *E. alabamensis* respectively in that daughter formation is initiated internally but differs from *Toxoplasma* in that the daughters protrude from the surface of the parent when partially formed and formation is completed by protrusion from the surface in a way similar to "normal" schizogony.

The process of asexual multiplication described in this paper is similar to the results reported by *Sheffield* (1970), *Colley & Zaman* (1970) and *Piekarski et al.* (1971). Like them we did not study the other forms described in a light microscope study of the early asexual division by *Dubey & Frenkel* (1972).

Piekarski et al. (1971) have proposed the term *endopolygeny* for this process, a term used previously by *Vivier* (1970) to describe the process by which endozoites of *Toxoplasma* undergo asexual multiplication in peritoneal exudate to give rise to more than two daughters. However, the terminology used to describe the various types of asexual division is in need of revision.

In comparing endopolygeny with endodyogeny, "normal" schizogony and the asexual process described by *Roberts et al.* (1970) there are points of similarity between each process, and as a result of further study it may transpire that these processes are variations of the same mode of development.

To explain the experimental findings that large numbers of mature schizonts contain an odd number of daughters it was proposed by *Ickerman & Cox* (1967) after studying *Plasmodium vinckei* that the nuclei did not divide simultaneously. A similar theory was proposed by *Lee & Millard* (1971) for *Eimeria praecox*. This theory is not admissible in the case of the endogenous forms of *Toxoplasma* where it was thought by *Colley & Za-*

man (1970) that each nucleus divided between two daughters, which would always produce even numbers of daughters. From this study it is possible that odd and even numbers of daughters can arise as the result of each nucleus giving rise to either one *or* two daughters.

The microgametocytes at the end of the phase of nuclear division are similar to those described for *Eimeria spp* by *Cheissin* (1965) *Hammond et al* (1967 & 1969) *Snigirevskaya* (1969a) and *McLaren* (1969) and reviewed by *Scholtyseck et al* (1972). The centrioles are found between the nuclei and the periphery as is the case found in *Eimeria spp* (*Scholtyseck et al.* 1972).

The partial disintegration of the inner membrane of the pellicle during gamete formation as observed by us confirms the report of *Pelster & Piekarski* (1971) for *Toxoplasma* in being similar to that seen in *Eimeria spp* by *Scholtyseck et al* (1972). There is a thickened membrane under the enclosing membrane in the area where the microgamete is protruding which is similar to that described for *Eimeria spp* and *Toxoplasma* (*Scholtyseck et al.* 1972).

The microgamete has a nucleus and mitochondrion similar to those observed by *Pelster & Piekarski* (1971). The presence of two flagella confirms reports by *Colley & Zaman* (1970) and *Pelster & Piekarski* (1971).

The latter authors observed 5 microtubules running longitudinally; we have only seen 4 in mature microgametes but between 2 and 5 microtubules have been observed in earlier stages of development. Four microtubules were also seen in *Eimeria maxima* (*Scholtyseck et al.* 1972) and in *Sarcocystis* (*Vetterling et al.* 1972). These 4 microtubules in *Toxoplasma* were found in a position which is occupied by a third flagellum in certain *Eimeria* species. The appearance in some sections of a unit membrane with an underlying osmiophilic layer enclosing the anterior part of the microgamete has not been reported in microgametes of *Eimeria spp* but 2 unit membranes have been reported for the microgametes of *Eucoccidium dinophili* (*Bardele* 1966).

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ABBREVIATIONS IN PLATES

B – Basal Body
C – Conoid
CH – Chromatin
DB – Dense Body possibly E-body
DR – Duct of Rhoptries (= Paired Organelles)
ER – Endoplasmic Reticulum
FL – Flagellum
G – Golgi Body
HN – Host Cell Nucleus
IM – Inner Membrane of Pellicle of Developing Merozoite
IV – Invagination
L – Lipid
M – Merozoite
MI – Mitochondrion
MN – Micronemes
MP – Micropore
MV – Microvilli of Host Cell
N – Nucleus
NU – Nucleolus
NR – Electron Transparent Portion of Nucleus
OL – Osmiophilic Layer
P – Perforatorium
PG – Polysaccharide Granules
PL – Pellicle
PN – Pole of Nucleus
PV – Parasitophorous Vacuole
R – Rhoptries (= Paired Organelles)
T – Microtubules
UM – Unit Membrane

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A PLATE-DIFFUSION METHOD FOR THE DETECTION OF ANTISTAPHYLOLYSIN ACTIVITY OF HUMAN SERUM

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A plate-diffusion method designed for routine use for detection of antistaphylolysin activity of human serum is described. The test is performed in Petri dishes containing rabbit erythrocytes embedded in agar. The method is simple to perform, reliable and reproducible.

In spite of the low antistaphylolysin titres usually seen in staphylococcal infections (1, 2) requests from the clinic for antistaphylolysin determinations are common. Most diagnostic laboratories perform antistaphylolysin determinations as the only serological test in staphylococcal disease.

The present paper deals with a simplified method designed for routine use for the detection of antistaphylolysin activity of human serum.

The aim of the study was to develop a simplified screening method for the detection of antistaphylolysin activity and to compare it with a conventional tube method designed with a view to detecting 2 IU of antistaphylolysin/ml serum.

MATERIALS AND METHODS

Rabbit erythrocytes. 5 ml rabbit blood, drawn by puncture of the ear vein, was mixed with 5 ml of Alsever's solution. The erythrocytes were washed twice in Jensen's buffer (per L: KH_2PO_4 1.45 g, $Na_2HPO_4 \cdot 2H_2O$ 7.6 g, NaCl, 4.8 g) with the addition of 1 per cent bovine serum albumin.

For the pouring of plates, a suspension of erythrocytes was used which, if diluted 1:100 with albumin-supplemented Jensen's buffer, gave an absorbance of 0.35 ± 0.02 in Beckman Model C Colorimeter using the green filter.

Preparation of agar plates. Equal amounts of 2 per cent agar (Agar Noble, Difco) and Jensen's buffer without albumin were melted and mixed. The mixture was allowed to cool to 50° C ± 1 and was mixed with the erythrocyte suspension, one volume of the erythrocyte suspension and 24 volumes of the agar mixture. 35 ml of this mixture was poured into sterile plastic Petri dishes with an inner diameter of 13.5 cm on a horizontal table. Plates were allowed to solidify. Cylindrical holes, 2 mm in diameter, were punched by a metal cylinder, about 70 holes/plate.

Performance of the antistaphylolysin determinations. 9.5 µl of the serum to be examined was transferred by the aid of a Hamilton syringe, one sample/hole. Between each transfer the syringe was rinsed three times with physiological saline. The plate was incubated with the lid on for two hours at room temperature (2? C). Staphylolysin (Staphylokocktesttoxin No 35, 2? IU/ml, Statens Bakteriologiska Laboratorium, Solna, Sweden) was diluted 1:8 with Jensen's buffer with 1 per cent bovine serum albumin, and 9.5 µl was applied to each hole by a Hamilton syringe. The plate was incubated at 37°C with the lid on for two hours and the zones of haemolysis were read with the naked eye and compared to a standard consisting of four samples with known antistaphylolysin titres determined by the conventional tube-dilution method. The antistaphylolysin titres of the standard sera were < 2, 4 and 8 units/ml. The sera were also tested by the conventional tube-method used in the

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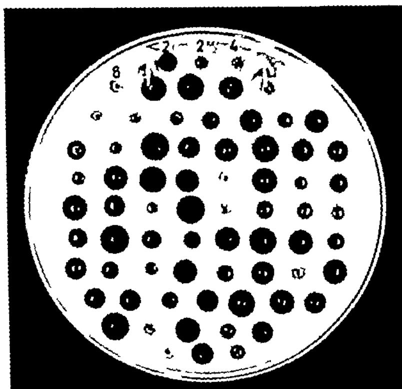


Fig. 1 Plate containing 66 human sera tested for antistaphylolysin activity. Four sera with known antistaphylolysin titres included (top row and sample far to the left in the second row)

laboratory and adjusted so as to detect 2 IU antistaphylolysin/ml.

Further the tube-method and the plate-diffusion method were compared as regards time and materials required for the performance of the tests.

RESULTS

Fig. 1 shows the outcome of 66 tested human sera from different patients together with four standard sera. The standard sera are seen in the three wells at the top of the plate and in the well situated far to the left in the second row from the top. The method was adjusted to give sera with 2 units of antistaphylolysin a small but distinct zone of haemolysis (second well from the left in the top row). Sera with 4 units/ml gave a hardly visible zone of haemolysis. Samples giving such narrow zones of haemolysis and samples giving no zone of haemolysis were titrated further using the conventional tube-dilution method.

Table 1 shows a comparison between 912 serum samples sent to the laboratory for antistaphylolysin determination and tested by the plate method and the routine tube-dilution method used in the laboratory. The tube method was adjusted so as to detect sera with antistaphylolysin activity of more than 2 units/ml. These sera were then titrated further. As appears from the table there was a high degree of correspondence between the two methods. By the tube method 107 sera were found to have titres of more than 2 units/ml. By the plate method, the corresponding figure was 98. Judged from the results of the tube method alone 21 sera were titrated further (Table 1 upper right square). Using the plate method these were considered to be $\leqq 2$ units/ml. When these sera were titrated further none was found to

have a titre of more than 4 units/ml. On the other hand, 12 sera were subjected to further titration if judged from the plate method alone (Table 1 lower left square). According to the tube method, all these had titres of 2 units/ml.

TABLE 1 *Comparison between Antistaphylolysin Titres Obtained by the Tube Dilution and the Plate Diffusion Method in 912 Human Sera*

		Tube dilution method	
		≦ 2	> 2
Plate diffusion method	≦ 2	793	21
	> 2	1	86

The reproducibility of the method was good. When 148 sera were tested on different occasions within one week, a difference of more than one titre step was never encountered in an individual sample.

Economic aspects. In this laboratory about 150 sera from different patients are tested for *antistaphylolysin activity every week*. Using the conventional tube method for screening one technician is occupied four hours/week. By the plate-diffusion method, the same work could be done in two hours. Plates containing rabbit erythrocytes could be stored for at least one day without negative effects.

The cost of the material for the plate diffusion method was *20–30* per cent of the cost of that used for the conventional tube method.

DISCUSSION

Zettervall described a method for the detection of antistaphylolysin in human serum based upon diffusion in agar (3). The method described by him has certain disadvantages *as regards its usability for routine* diagnostic purposes, such as the long diffusion time of antistaphylolysin material (>10 h), the incorporation of staphylolysin in the medium and the *need for treatment* of the plates with formalin before reading. In all these respects, the present method offers some advantages. The *total* time *interval* from the application of the serum sample to the reading of the result is 4 h if the present method is used, thus permitting further titrations *to be performed on the same* day as the screening. It should be noted that the figures given for the time required for the performance of *the test*, as well as those *referring* to the cost are influenced by many factors, depending on the individual laboratory. However, in our laboratory, the present plate-diffusion *method for* detection of antistaphylolysin in human sera has proven to be fast, reliable and technically simple to perform. Apparent drawbacks have *so far not* been encountered.

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STUDIES OF THE *TREPONEMA PALLIDUM* IMMOBILIZING ACTIVITY IN NORMAL HUMAN SERUM I A METHOD

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Taking advantage of the experiences gained with the TPI-test (*T pallidum* immobilization reaction by immune serum) a method for quantitation of *T pallidum* immobilizing activity in normal unheated human serum was developed on the basis of the kinetics and optimal conditions of this normal serum reaction *in vitro*. Some experiments concerning factors influencing the haemolytic complement activity (e.g. the SH-compounds of the TPI test medium) as well as the treponemal survival were also presented. As compared with the immune serum reaction, the normal serum reaction had a much shorter lag phase at high serum concentrations it was not measurable, i.e. <10 minutes, and the reaction rate was faster the reaction being almost completed within two hours. The length of the lag period increased with treponemal concentration and decreased with temperature. The reaction rate after the lag period appeared to be influenced only to minor degree within certain ranges, either by concentrations of serum or by the concentration of the treponemes. The temperature coefficient (Q_{10}) was found to range around two to three. The normal serum reaction was dependent on the ionic strength, being enhanced below and depressed above the ionic strength of 0 15 The optimal pH, tested within the range 6.8–7.8 was found to be 7.2–7 8. Mg^{++} in contrast to Ca^{++} had an enhancing effect on the normal serum activity the optimal final concentration of Mg^{++} ranging around 0 005 M. The normal serum immobilization reaction resembled in many respects the bactericidal and bacteriolytic reactions to be evoked by normal serum.

In a preliminary paper (*Hederstedt* 1962) it was reported that unheated human normal serum (NS) alone, without any addition of immune serum (IS) immobilized *Treponema pallidum* Later the author has been informed of a brief study of the immobilizing activity in NS by *Fribourg-Blanc* (1956) The results obtained in this study will be discussed in succeeding papers (*Hederstedt* to be published)

As distinguished from the *T pallidum* immobilization by IS (*Nelson & Mayer* 1949) the NS immobilization could not be brought about by heated serum to which guinea pig serum was added as source of complement. As in the IS immobilization reaction, complement and lysozyme appeared to take part in the NS reaction (*Hederstedt* 1963)

The observation that the majority of unheated human NS as well as sera from certain animals possessed this *T pallidum* immobilizing activity (*Hederstedt* 1963) raised questions concerning the nature of this activity and its relation to the well known activity of unheated NS on certain bacteria and

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TABLE 1 *Final Concentrations as well as Concentrations of Stock Solutions of the Constituents of the Nelson Medium and a Revised Medium used for Determinations of T pallidum Immobilizing Activity of Immune and Normal Serum Respectively*

Constituent	Nelson Medium		Revised Medium	
	Stock-solution	Final concentration	Stock-solution	Final concentration
Crystal, bovine Albumin	–	2.5 %		2.5 %
Dissolved in sodium chloride	0.15 M	0 075 M		
or in distilled water			0.0 M	0 0 M
Phosphate buffer (pH 7 1) Na_2HPO_4	0.10 M	0 0125 M	0.10 M	0 0125 M
KH_2PO_4	0 15 M	0 00472 M	0.15 M	0 00472 M
Sodium pyruvate	0 098 M	0.00152 M	0 098 M	0.00152 M
Sodium bicarbonate	0 15 M	0 00847 M	–	–
Cysteine	0.04 M	0 00126 M	–	–
Glutathione	0 04 M	0.00250 M	–	–
Sodium thioglycollate	0.1314 M	0.01574 M	–	–
Sodium chloride	0.15 M	0.00863 M	–	–
Magnesium chloride			1.0 M	0.01 M

viruses. Questions concerning its possible immunological role in syphilis as well as its importance for the outcome of the *Treponema pallidum* immobilization (TPI) test (*Nelson & Diesendruck* 1951) were also raised and considered important to study

Taking advantage of the experiences obtained from the well known TPI test, with IS a method for quantitative determination of the *T pallidum* immobilizing activity in human NS was developed on the basis of the present study of the kinetics and optimal conditions of the NS immobilization reaction *in vitro*

MATERIALS AND METHODS

The treponemal strain. The virulent Nichol strain of *Treponema pallidum* used throughout these experiments was received from the Statens Seruminstitut, Copenhagen, in 1955 and since then it has been perpetuated by intratesticular inoculation of rabbits.

The normal sera (NS) Blood was drawn from healthy persons without a history of syphilis. The blood was allowed to clot for two hours at room temperature. Serum was separated by centrifugation and stored at –60 C. An aliquot of each NS was inactivated (56 C, 30 min.) and stored at –20° C. Prior to each test, the latter serum was re-inactivated (56 C, 10 min.) NS was non-reactive in the Wassermann reaction and the TPI test.

The diluent The sera were diluted in a veronal NaCl buffer containing 0 00015 M Ca^{++} and 0 0005 M Mg^{++} pH 7 4 (*Kabat & Mayer* 1961)

The basal medium Originally the medium used for extraction of treponemes from rabbit testes was the same as that used in the TPI test, the Nelson medium (Table 1) However if not otherwise stated, a revised medium composed of bovine albumin dissolved in distilled water phosphate buffer sodium pyruvate and magnesium chloride (Table 1) was used in the experiments.

The treponemal suspension Rabbits were inoculated intratesticularly with 1 ml of the treponemal suspension (4.5 × 10 –10⁸ treponemes/ml) in each testicle and the testicles were collected six to eight days later The rabbits were killed by exsanguination from the heart. The testicles were cut in slices and added to 10 ml of basal medium. The treponemes were extracted from the tissue by shaking for a few minutes and separated from tissue debris by repeated centrifugation at 300 G. In each experiment, the treponemal concentration was estimated in 0.005 ml of treponemal suspension under a 18 × 18.2 mm cover glass at 500 × by counting the number of treponemes in 25 microscopic fields. An appropriate amount of basal medium was added to dilute the treponemal suspension used in the experiment so that it contained about 4 5 × 10^6 treponemes per ml (5 treponemes per field)

The test procedure In the course of investigation, the test procedure was frequently revised but unless otherwise stated the experiments were performed according to the following procedure

In a test-tube 8 × 80 mm, aliquots of NS (undiluted or diluted) and treponemal suspension (4.5 × 10^6 treponemes per ml) in a total volume of 0.5 ml were mixed For each tube with

heated NS, a control tube containing inactivated NS was set up. Unless otherwise stated, the mixture was incubated in an anaerobic jar filled with a gas mixture of 95 per cent N and five per cent CO_2 for 120 minutes at 35 C. NS, treponemal suspension and gas mixture were prewarmed to 35 C. I experiments where the test mixtures were incubated t different temperatures, anaerobic conditions were maintained by pouring a layer of paraffin oil containing 0 1 per cent of an oil soluble anti-oxidant, 2 6-ditertbutyl paracresol, over the test mixtures (*Weber* 1960) In fact, sufficient numbers of prewarmed gas mixtures were not available in these experiments.

Reading was performed by darkfield microscopy at 500 × and 50 treponemes per tube were counted. The percentage of immobilized treponemes was calculated by comparing the number of motile treponemes in unheated and inactivated NS (*Nelson & Diesendruck* 1951) The presence of at least 90 per cent motile treponemes in inactivated NS was set as a criterion of the validity of an experiment.

In the kinetic experiments t the end of the incubation periods, the test tubes were chilled in ice water for 20 seconds to about 15 C to slow down the reaction rate. Subsequently reading was performed at room temperature.

For quantitative testing, two-fold dilutions of the NS were made in two separate two-fold step series, the initial NS dilutions being undiluted and 1 1.4 respectively The pipette was changed for each dilution. The NS titre was recorded as the reciprocal of the highest dilution step causing ≥50 per cent immobilization or as the negative logarithm of the serum dilution causing 50 per cent immobilization of the treponemes when the per cent immobilization was plotted against the negative logarithm of serum dilution. In the latter case the titre was calculated on the basis of the best fitted straight line, usually that between three points in the semilogarithmic plot.

Qualitative controls for excess haemolytic activity of complement were made on all tubes by adding 0.1 ml of a two per cent suspension of sensitized sheep blood cells and incubating for 30 minutes, 35 C. Reading was performed after 16 to 20 hours at 4 C Excess of haemolytic C-activity was held to be demonstrated if there was 100 per cent haemolysis i.e. no visible blood cells at the bottom of the test-tube.

RESULTS

Before presenting the experiments, some facts of principal importance for a better under standing of the present investigation will be pointed out

Firstly *Treponema pallidum* is very sensitive to oxygen and has not yet been cultivated *in vitro* However the treponemes can be kept alive for a few days if incubated in a CO_2–N_2 atmosphere in a specific survival medium, the Nelson medium, containing i.a. oxygen-reducing SH-compounds (Table 1)

TABLE 2. *Effect of the SH-compounds of the T ponemal Suspension on the Imm bilizing Capacity and Haemolytic C-activity of a Normal H man Serum. Ionic Strength of the Two Media Was 0 15 the Test Mixture Was Incubated in CO –N Atmo phere for 90 minut s. The Tr ponemes Used in the Experiment wer Derived from one and the ame Rabbit Testicle*

Serum concentration	SH-containing treponemal suspension		SH free treponemal suspension	
	Per cent immobilization	Residual complement	Per cent immobilization	Residual complement
0.50	88	+	93	+
0.36	64	+	96	+
0.25	48	+	96	+
0.18	12	—	88	+
0 13	12	—	52	+
0.09	4	—	21	+
0 07	0	—	4	+
0.04	6	—	4	+
0 03	4	—	8	+
				—

Residual haemolytic complement activity was tested qualitatively (100 per cent haemolysis denoted +)

TABLE 3 *Number of Sera Showing less than 90 per cent Treponemal Survival in Revised Medium (Table 1) after One Two Three and Four Hours' Incubation in Air*

Type of incubation	Serum concentration	Incubation period (hour)			
		1	2	3	4
I	0.5	9	10	10	10
	0.03	10	10	10	10
II	0.5	1	10	10	10
	0.03	2	10	10	10
III	0.5	0	1	10	10
	0.03	0	2	10	10
IV	0.5	0	0	1	10
	0.03	0	0	1	10
Control	0.5	0	0	0	0
	0.03	0	0	0	0

Note Ten inactivated human sera at the concentrations of 0.5 and 0.03 were tested. Incubations were performed in four different ways:

I Incubation, air 35 C.

II pre-incubation, N_2–CO_2 for five minutes + incubation, air 35 C.

III pre-incubation, N_2–CO_2 for 120 minutes + incubation, air 35 C.

IV pre-incubation, N –CO_2 for 120 minutes + incubation, air 22 C.

Control: incubation N_2–CO_2 35 C in Nelson medium.

Secondly in preliminary experiments, the immobilization reaction evoked by human NS was found to be complement dependent just like the reaction evoked by IS but more rapid than the immobilization caused by immune antibodies. While high titre NS immobilized 100 per cent of the treponemes within 30 minutes, high titred IS required four to six hours to accomplish the same effect.

It was considered of importance to present experiments concerning factors influ ...ing the haemolytic complement activity ... well as the treponemal survival prior to the results obtained in studies of the kinetics and optimal conditions of the immobilization reaction.

Factors Affecting the Haemolytic Complement Activity (SH-compounds Treponemal Suspension Incubation Period)

Table 2 illustrates the inhibiting effect of the SH-compounds of the treponemal suspension on the haemolytic C-activity as well as that on the immobilizing capacity of a human NS

Treponemal suspensions from different rabbits exerted different C-inhibiting actions. In the same NS as that used in the experiments illustrated in Table 2, the residual C-activity demonstrated at the lowest serum concentration varied between 0.13 and 0.03 if mixed with treponemal suspensions (freed of SH-compounds) from 10 rabbits.

The decay of haemolytic C-activity caused by an incubation period of two hours at 35 C was found to be insignificant as compared with the decay brought about by the SH-compounds and the treponemal suspensions.

Thus, to assure that complement would not be a limiting factor for the determination of treponemal immobilization caused by unheated NS it was considered necessary to eliminate the SH-compounds from the basal medium

Treponemal Survival

By eliminating the SH-compounds from the basal medium the mobility period of the treponemes was shortened A further omitting of sodium bicarbonate did not appear to im-

pair treponemal mobility which would be impaired if any other ingredient of the medium (Table 1) were excluded.

Using the revised medium after incubation in air at 35 C (Table 3) treponemal mobility in 10 inactivated NS in concentrations of 0.5 and 0.03 was significantly impaired within one hour The treponemal survival in air was prolonged if the mixture of treponemes and serum was pre incubated in N_2–CO_2 atmosphere at 35 C. After such pre incubation for 120 minutes the subsequent mobility period in air at 35 C would be around two hours and if the air temperature was lowered to 22 C, reliable survival of the treponemes for three hours was noticed in the majority of sera.

Thus, after two hours' incubation of the test mixtures in N_2–CO_2 at 35 C, it is possible to examine these microscopically for at least two hours, without any occurrence of non-specific immobilization of the treponemes.

Immobilization at Various Serum Concentrations

Equal amounts of treponemal suspension and unheated human NS at various dilutions

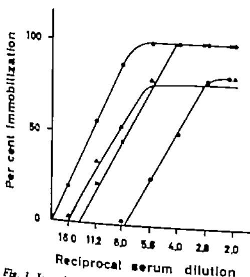


Fig. 1 Immobilization-serum concentrations curves of one normal human serum tested in four different experiments.

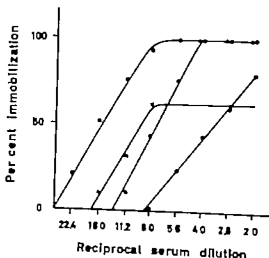


Fig 2 Immobilization-serum concentration curves of four normal human sera tested in one and the same experiment.

were mixed and incubated for two hours in N_2–CO_2 atmosphere at 35 C. For each serum dilution, the percentage of immobilized treponemes was computed and plotted against log serum concentrations.

The immobilizing titre of the NS i.e. the log serum concentration causing 50 per cent immobilization, was determined in total on 21 testing days (see also under "Reproducibility experiments" Table 7) In addition to the finding that the NS immobilizing titre varied much (Table 7) the results of these experiments revealed that the shape of the immobilization curves varied somewhat from experiment to experiment. This variation is illustrated by four selected curves in Fig. 1 In two out of 19 technically successful experiments, the immobilization did not continue to the 100 per cent level in spite of increasing serum concentrations (Fig 1)

Similar variations were found on simultaneously performed tests of reactive sera from 20 healthy persons. In Fig. 2, the curves of four of these NS illustrate the variations in shapes of the immobilization curves in sera from different persons. In a few NS, some of the treponemes seem to withstand the immobilizing activity In one of the NS as much as 40 per cent remained mobile, independent of increasing serum concentra

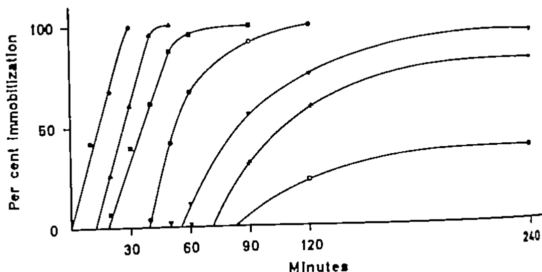


Fig 3 Per cent immobilization by a normal human serum at various reaction times and serum concentrations. Final serum concentration from left to right 0.5 0.36 0.179 0.0893 0.0625, 0.0H7 and 0.0313

tion Every curve, however appeared to have a steep and linear course around 50 per cent immobilization.

The slope value in the probit diagram—regression of the probit of percentages of immobilized treponemes upon the logarithm of serum concentration if determined in 20 unheated human NS, was found to range between 6 7–5.0 with a mean of 6.0

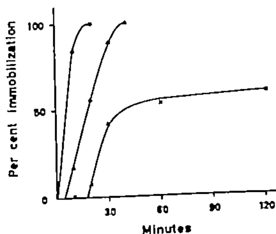


Fig 4 Per cent immobilized treponemes in one normal human serum at various reaction times and at three different treponemal concentrations: 9.0 × 10⁵ 9.0 × 10⁶ and 9.0 × 10⁷ treponemes per ml (illustrated from left to right)

Immobilization at Various Incubation Times and Serum Concentrations

The kinetics of treponemal immobilization brought about by various concentrations of an unheated human NS was calculated from a series of experiments one of which is illustrated in Fig. 3 At the highest serum concentrations there was no measurable lag phase, i.e. less than 10 minutes. At the other serum concentrations, the reaction had a lag period which varied from around 10 minutes to 60–90 minutes, inversely related with the serum concentration.

Within 30–120 minutes, 100 per cent of the treponemes were immobilized at the higher serum concentrations and the slope values of the reaction curves around the 50 per cent immobilization level was of approximately the same magnitude In the lower serum concentrations, the overall reaction rate was slower after two hours it had almost stopped, without having reached the 100 per cent level.

Immobilization at Varying Treponemal Concentrations

To study the influence of treponemal concentration on the immobilization reaction

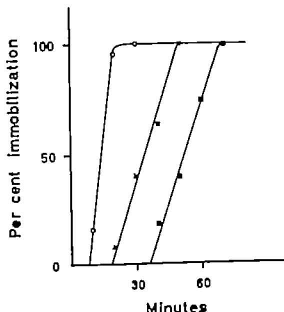


Fig 6 Per cent immobilized treponemes in one human serum, recorded every tenth minute at three different temperatures 35 (O) 30 (×) and 25 C (■)

37 C the treponemes showed less than 90 per cent survival. No immobilization was de monstrated within two hours at 19 C.

The kinetics of the reaction were studied in five other experiments at three different temperatures 25 30 and 35 C One of these experiments is illustrated in Fig 6. The lag period varied inversely related with the temperature.

The temperature coefficient, Q_{10}, for the reaction rate within the temperature ranges 25–35° C, varied in the five experiments be-en 1.2–3 6, averaging 2.7

The practical consequences of the present study on the temperature dependence of the NS immobilization reaction might be summarized as follows The lowest temperature giving maximal immobilization within two hours, 35 C, would be the incubation temperature of choice for a quantitative test procedure. The immobilization can be arrested by chilling the test mixture to 15 C. Moreover if optimal immobilization were to be achieved the serum, the treponemal suspension and the gas mixture (N_2–CO_2) had to be separately prewarmed to 35 C.

Immobilization at Varying Ionic Strength

In the experiment illustrated in Fig. 7 the effect of ionic strength within the range 0 10–0.27 on the immobilization at varying serum concentrations was studied An ionic strength of less than 0 04–0 05 in the basal medium was incompatible with reliable treponemal mobility At low serum concentrations, optimal immobilization seemed to occur within the ionic strength range of 0.10–0.14, while the degree of immobilization would be reduced at 0 16 At ionic strengths of >0.13 it was observed in high titre sera that a small part of the treponemes remained mobile regardless of increasing serum concentration.

Approximately the same results were obtained if the ionic strength was varied and different concentrations of the phosphate buffers were used (Na_2HPO and KH_2PO_4) These results are in support of the theory that the observed effects on immobilization may be due to the ionic strength itself and not to osmotic pressure or to the action of specific ions.

According to the above results, the final ionic strength at the quantitative determina-

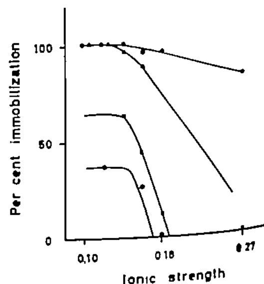


Fig 7 Immobilization at various ionic strengths, 0.10–0.27 and serum concentrations, 0.5 (●) 0.0893 (Δ) 0 0447 (×) and 0 0313 (O)

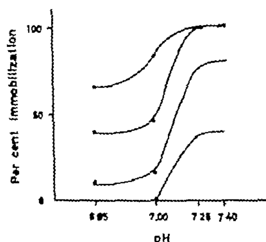


Fig. 8. Immobilization at various pH, 6.65–7.40 and serum concentrations, 0.179 (×) 0.125 (○) 0.0893 (●) and 0.0625 (△)

tion of the NS immobilization should be between 0.10–0.14.

Immobilization at Varying pH

In the above study of the influence of ionic strength on immobilization, the pH in the test medium and the diluent was adjusted to 7.4. In the reaction mixtures the pH was measured at the beginning and at the end of the experiments. At the high serum concentrations, 0.5 and 0.36, the pH was found to be 7.6–7.8 at the start and 7.5–7.6 at the end of the experiment. At serum concentrations of 0.25 and below the pH was found to be more constant and within the range of 7.4–7.5.

Immobilization caused by NS can be demonstrated only in relatively high serum concentrations, rarely <0.0313 (to be published). The pH dependence in the present experiments was studied in serum concentrations of 0.179–0.625.

Two different buffers, $Na_2HPO_4 + KH_2PO_4$ and Tris-HCl phosphate were used at varying pH as constituents of the medium and as serum diluents. The total ionic strength ranged at 0.12 in all experiments. The pH of the test mixture was measured at the start and at the end of the experiments.

As seen in Fig. 8, the percentage of immobilized treponemes within the pH range 6.2–7.4 using $Na_2HPO_4 + KH_2PO_4$ as buffer was highest at 7.2–7.4. Using Tris-HCl phosphate buffer within the pH range 7.2–8.6, no variations in sensitivity of the reaction were observed at 7.2–8.0. Thus, the variation of pH within the range 7.5–7.8 found to occur at high serum concentrations, >0.25 does not seem to influence the sensitivity of the reaction. In most experiments treponemal mobility was impaired at pH ≤6.6 and ≥8.0.

In the quantitative test procedure the medium was adjusted to pH 7.4 with a buffer containing $Na_2HPO_4 + KH_2PO_4$. A veronal buffer of pH 7.4 was used as serum diluent.

Immobilization at Varying Concentrations of Calcium and Magnesium

The concentrations of Ca^{++} and Mg^{++} in the reaction mixture were varied by adding these salts to either the serum diluent or to the treponemal medium, or to both of these components.

Table 4 records the immobilizing titre of a human NS diluted in three different diluents (1) 0.15 M NaCl, (2) veronal buffer containing 0.00015 M Ca^{++} and 0.0005 M Mg^{++} i.e. concentrations found to be optimal

TABLE 4. *Immobilizing Titre and Residual C-activity of a Human Serum Diluted in Three Different Diluents*

Serum diluent	Immobilizing titre	Residual C-activity
0.15 M NaCl	16.0	22.4
Veronal buffer (0.00015 M Ca^{++} 0.0005 M Mg^{++})	22.4	44.8
Veronal buffer (0.0072 M Ca^{++} 0.0024 M Mg^{++})	11.2	44.8

TABLE 5 *Immobilizing Titres as well as Residual C-activity of a Normal Human Serum Mixed with Treponemal Suspensions Containing 0.005 M EDTA and Various Concentrations of* Mg^{++} *(0.005–0.16 M) or* Ca^{++} *(0.01–0.08 M). Controls with Treponemal Suspensions without Added* Mg^{++} *or* Ca^{++} *with EDTA and without EDTA Added. Final Ionic Strength of the Reaction Mixtures Is also Given*

Molarity added to treponemal suspension: EDTA	Molarity added to treponemal suspension: Mg^{++} or Ca^{++}	Final ionic strength of reaction mixture	Mg^{++}: Immobilizing titre	Mg^{++}: Residual C-activity	Ca^{++}: Immobilizing titre	Ca^{++}: Residual C-activity
0.005	0.005	0.15	<2.0	<2.0		
0.005	0.01	0.15	5.6	16.0	<2.0	<2.0
0.005	0.02	0.15	16.0	22.4	4.0	16.0
0.005	0.04	0.16	22.4	22.4	—*	—†
0.005	0.08	0.22	11.2	22.4	—	—†
0.005	0.16	0.34	4.0	5.6		
0.005	0.0	0.15	<2.0	<2.0		
0.0	0.0	0.15	8.0	16.0		

* The reaction mixture was toxic for the treponemes.

† Heavy precipitates of calcium salts.

TABLE 6. *Immobilizing Titres as well as Residual C-activity of a Normal Human Serum, Mixed with Treponemal Suspension to which Were Added various Concentrations of* Mg^{++} *(0.025–0.08 M)*

Molarity Mg^{++} added to treponemal suspension	Final ionic strength of reaction mixture	Immobilizing titre	Residual C-activity
0.0	0.15	8.0	16.0
0.0025	0.15	16.0	22.4
0.005	0.15	16.0	22.4
0.01	0.15	22.4	22.4
0.02	0.15	22.4	22.4
0.04	0.16	22.4	22.4
0.08	0.22	16.0	11.2

for complement dependent immune haemolysis, (*Kabat & Mayer* 1961) or (3) veronal buffer containing 0.0072 M Ca^{++} and 0.0024 M Mg^{++} i.e. concentrations present in undiluted human NS (*Wardlaw & Pillemer* 1956) Ca^{++} or Mg^{++} were not added to the medium.

The highest immobilizing titre of the NS 22.4 was found when the veronal buffer containing 0.00015 M Ca^{++} and 0.0005 M Mg^{++} was used. In the presence of physiological concentrations of Ca^{++} and Mg^{++} the NS titre (11.2) would be even lower than the titre (16.0) obtained when 0.15 M NaCl was used as diluent. The residual C-activity of the reaction mixtures containing the two veronal buffers, however was greater (44.8) than that observed when 0.15 M NaCl was used as diluent (22.4).

Various concentrations, 0.0025 0.005 and 0.01 M of the chelating agent ethylenediminetetra acetate (EDTA) were added to the treponemal suspension. Different human NS were repeatedly tested for immobilizing capacity. Veronal buffer (0.00015 M Ca^{++} and 0.0005 M Mg^{++}) was used as serum diluent. In all the experiments 0.005 M was the lowest concentration of EDTA depleting the sera of immobilizing activity as well as the haemolytic C-activity.

The immobilizing activity of a human NS mixed with treponemal suspensions contain-

TABLE 7 *Analysis of Variance in Reproducibility Experiments*

Variation	Sum of squares	Degrees of freedom	Mean squares	V^2-test	Standard deviation	$3\sqrt{2}\times\sigma$
Between duplicate tests	0.0112	19	0.000589	31.73	0.024	0.103
Between tests carried out on different days	0.3563	18	0.01869		0.095	0.404

ing 0.005 M EDTA, was recorded after addition of various amounts of 1.0 M $MgCl_2$ or $CaCl_2$ to the suspensions. As seen in Table 5, the immobilizing titre increased with increasing concentrations of Mg^{++} up to 0.04 M. At higher concentrations, decreasing titres were recorded. However, the ionic strength of the reaction mixtures containing 0.02 M Mg^{++} or Ca^{++} was increased by the addition of these salts. Thus, at the concentration of 0.08 M Mg^{++} the final ionic strength of the reaction mixture would be 0.22, which from the preceding experiments is known not to give optimal immobilization. In some experiments, treponemal survival was impaired at Mg^{++} concentrations of ≥ 0.008 M. Addition of Ca^{++} reactivated the immobilizing capacity of serum, but not to the same extent as adding of Mg^{++}.

In another experiments, 0.0005, 0.005, 0.01 or 0.02 M Ca^{++} as well as 0.02 or 0.04 M Mg^{++} were added to 0.05 M EDTA treponemal suspensions. The simultaneous addition of Ca^{++} and Mg^{++} did not further increase the potentiating effect of the immobilizing NS activity to be caused by Mg^{++} alone.

Finally, the effect of adding various concentrations of Mg^{++} to treponemal suspensions in the absence of EDTA on the immobilizing NS activity was studied. As seen in Table 6, the immobilizing capacity of NS was increased by the addition of Mg^{++} within the range of 0.01–0.04 M Mg^{++}.

According to the results obtained in the above experiments, the treponemal suspension medium used for the determination of immobilizing activity in human NS should contain 0.01 M Mg^{++}, i.e. the final molarity of added Mg^{++} of the reaction mixture being approximately 0.005.

Reproducibility Experiments

Using the test procedure established on the basis of results obtained in the present investigation, the immobilizing titre of a given unheated NS tested in duplicate was determined in 21 consecutive experiments (on different days). Two of the experiments were unsuccessful because of poor treponemal mobility in inactivated serum. If $3\sqrt{2} \times \sigma$ were chosen as the level of significance for titres determined on one and the same day and on different days, a difference of 0.10 and 0.40 log units, respectively, would be statistically significant (Table 7).

DISCUSSION

The main purpose of the present experiments was to work out a method for quantitative determination of the *T. pallidum* immobilizing capacity of NS.

A comparison of the results obtained in the present study of the kinetics and the optimal conditions of this immobilization reaction in relation to corresponding data on IS immobilization as well as other antimicrobial reactions caused by NS, might give some information about i.a. the factors participating in the NS immobilization.

The observation that human NS heated at 56° C for 30 minutes fully lost its immobilizing capacity suggested that this activity in the NS, like the IS immobilization reaction, was complement-dependent. In the IS reaction, undiluted guinea pig serum, most

often in great excess, is the source of complement. In the NS reaction, the reacting serum s own complement is the only source. The content of haemolytic complement in human serum, using rabbit antisheep cell serum s own complement is the only source. that in guinea pig serum. To avoid complement being the limiting factor in the NS reaction, the complement inhibiting SH-compounds (*Cushman et al.* 1957) constituting a part of the basal medium used in the TPI test, had to be omitted. In fact, in the presence of SH-compound only 24 per cent of the human NS tested caused more than 50 per cent treponemal immobilization (*Hederstedt* 1962) as compared with 84 per cent if the SH-compounds were omitted (to be published)

By omitting the SH-compounds, the treponemal survival period would be markedly shortened. However the present study of the NS reaction revealed obvious differences as compared with the IS reaction. Thus, the NS reaction rate was faster and the reaction was almost arrested within two hours. During this period, the treponemes survived even in the SH-free medium. To keep the ionic strength of the medium as low as possible (see below) bicarbonate was also omitted. Thus, the buffering capacity of the reaction mixture (medium and serum) was still sufficient to keep the pH constant during the incubation period. Therefore it was not necessary to exclude the CO_2 from the incubation atmosphere which by *Nelson* (1948) was found to have a benefical effect on the treponemal survival.

In high titre NS at high serum concentrations, a lag phase if any was too short to be demonstrated, <10 minutes. At lower serum concentrations there would be a definite lag phase the length of which was inversely correlated with the serum concentration. The reaction rate, on the other hand within those serum concentrations causing 100 per cent immobilization appeared to be independent of the serum concentration. This observation is in agreement with the results obtained in studies of the kinetics of the IS reaction by *Nelson & Diesendruck* (1951) In that reaction, however an initial lag period covered at least two to four hours. The reaction was not arrested within 18 hours, the length of the incubation period generally adopted in the TPI test. Either very short or no lag periods were found for the NS collytic reaction (*Davies & Wedgewood* 1965) which is dependent on complement as well as lysozyme (*Wardlaw* 1962)

The slope of the NS concentration—immobilization curve in the probit diagram was found to range around 6.0, which is twice as high as the corresponding value of the IS immobilization (around 3.0) and somewhat lower than the slope denoting immune haemolysis which ranged around 7.0 (*Muschel* 1957) The two-fold dilution steps generally accepted for the determination of IS titres (*Nelson & Diesendruck* 1951) were not close enough for the quantitative determination of immobilizing activity caused by NS It was necessary to plot at least three values between 10 and 90 per cent on the immobilization curve For practical reasons, however and with a view to reading, it was desirable to have as few dilution tubes as possible. Accordingly in the present investigation, serum was diluted in two separate two-fold dilution-step series.

The study of the influence of varying concentrations of treponemes on the immobilization reaction revealed that the sensitivity was significantly reduced at the highest concentration. This was noticed even if complement was added in excess to the reaction mixture, thus suggesting that the antigen exceeded an immobilizing antibody in serum. Kinetic studies revealed that the length of the lag period increased with increasing treponemal concentrations. It should be noticed, however that increasing concentrations of treponemes in the reaction mixtures used in the present experiments implied that there would be increasing amounts of unavoidable coexisting material from rabbit testicle tissues In a following paper (*Hederstedt* to be published) it will be reported that rabbit testicle tissues have an antigen in common with *T*

pallidum and that the tissues used as sorbent are found to eliminate the immobilizing capacity of normal serum. Thus there is great probability that the different influence on the immobilization reaction to be noticed when varying concentrations of treponemes were used was accentuated in the presence of the rabbit testicle material.

The sensitivity of the IS reaction was found to vary inversely to the treponemal concentration (*Miller et al.* 1958). In the latter experiments, however, the influence of treponemal concentration on the IS reaction was tested only within a rather limited range and the treponemal suspensions to be tested contained 1.5 or 15 treponemes per microscopic field.

The studies of the effect of temperature on the NS immobilization showed that the sensitivity of the reaction increased with increasing temperature within the range of 19°–35 C. At 15 C, no immobilization could be demonstrated even after 18 hours incubation. At 37° C, treponemal survival would be impaired. The temperature dependence of the NS immobilization seemed to resemble that of the IS reaction within the range tested, 25–40 C (*Nelson & Mayer* 1949). Our experiments revealed that IS immobilization occurred after 18 hours incubation at 19° C while the treponemes even in high titre sera were not immobilized by incubation at 16 C for 66 hours (unpublished data). Immune haemolysis takes place at 0° C, if the concentration of antibody used to sensitize the erythrocytes is higher than optimal, i.e. optimal as determined by the usual amboceptor titration at 37° C (*McVicker & Wernfeld* 1959). The lysozyme dependent normal serum colilysis could not be demonstrated at 0 C (*Wardlaw* 1962) and the properdin-dependent immuno-inactivation of *Toxoplasma gondii* did apparently not occur below 20 C (*Strannegård* 1967).

The temperature coefficient (Q) of the normal serum calculated from the reaction rates of the temperature interval 25–35 C varied greatly from experiment to experiment and it was not considered advisable to make any thorough analysis of the kinetics. However, the apparently insignificant influence on the reaction rate of varying serum concentrations as well as of varying numbers of treponemes in the reaction mixture together with a Q_{10} probably around two to three suggest that the normal serum immobilization is a reaction of low order. The inactivation of coli T2 phage by normal human serum (*Kallings* 1961b) as well as the bactericidal action of human NS on gram-negative bacteria (*Davies & Wedgewood* 1965) are other reactions of low order.

The treponema immobilizing activity of NS appeared to vary inversely related with the ionic strength. The relation between degree of immobilization and ionic strength however was not found to be linear. Small variations of ionic strength, around 0.15 caused relatively great variations in the degree of immobilization (Fig. 7). Moreover at an ionic strength of >0.15 in high titre sera, part of the treponemes remained mobile regardless of increasing serum concentration (Table 2, Fig. 7). This inhibition of the reaction was almost invariably seen early in the present investigation, where more than one hundred NS at the ionic strength of 0.15 were tested. Reducing the salt concentration makes the electrostatic attraction between treponemes and reactive molecules stronger and facilitates collision with each other which may explain why this effect was rarely seen at the ionic strength of 0.11. The phenomenon might be analogous to the persistent surviving fraction observed in the inactivation of coli T2 phage by NS (*Kallings* 1961a). However, in the phage inactivation the phenomenon was apparently not overcome by lowering the ionic strength of the reaction mixture.

Another phenomenon was a "disappearance" of the treponemes, sometimes observed at ionic strengths of ≥0.15 as well as at high Ca^{++} concentrations. Under the microscope it appeared as if the treponemes were contracted to clumps during the immobilization phase. The phenomenon might be consistent with the finding by *Noguchi* (1912)

and *Hardy* (1960) that aberrant forms of treponemes occur under unfavourable environmental conditions. Optimal haemolytic complement activity (*Wardlaw* & *Walker* 1963 *Rapp* & *Borsos* 1963) and NS collilysis (*Wardlaw* 1962) were found at ionic strengths of 0 055–0 10

The optimum range for lysis of *Micrococcus lysodeikticus* by hen egg white lysozyme was found at ionic strengths of 0 025–0 12 (*Smolelis* & *Hartsell* 1952) whereas the corresponding range for lysozyme from tissue fluid of syphilitic rabbit testes has been reported to be 0.25–0 90 (*Metzger* & *Kopéc* 1967) The great difference between these two ionic strength optima supports the finding that lysozyme isolated from different species differ markedly in their enzymic activity chemical composition and physical properties (*Jollès* 1964) Any information concerning the ionic strength optimum of human lysozyme is apparently not available.

The author has not found any publications concerning the effect of ionic strength on IS immobilization. In preliminary experiments, an immobilization optimum in the vicinity of 0 15 was obtained while a significantly lower activity was demonstrated at the ionic strength of 0 12 (unpublished data)

The method by which to study the effect of varying hydrogen ion concentrations on treponemal immobilization by NS was technically complicated and the effect could be studied only within a relatively small pH range, from 6.8 to 7 8 (*Nelson* 1948) Outside this pH range, the treponemal survival period was too short to make any reliable determinations. NS exerted the highest immobilizing activity within the range pH 7.2–7.8

The influence of pH on the IS immobilization has apparently not been studied

Immune haemolysis caused by human complement was found to have a pH optimum around 6.5 while guinea pig complement did not seem to be very sensitive to pH alterations within the range from 6.0 to 8.0 (*Yachnin* & *Ruthenberg* 1965) Bacteriolytic activity produced by egg white lysozyme as well as by lysozyme from rabbit tissue fluid had an optimum at pH 6.0-6.6 (*Smolelis* & *Hartsell* 1952 *Metzger* & *Kopéc* 1967) The only available data on the pH dependency of human serum lysozyme, is an optimum pH of 5 0–5 1 using glycol chitin instead of *M lysodeikticus* as substrate (*Lundblad* & *Hultin* 1966) Preliminary unpublished experiments using *M lysodeikticus* as substrate revealed that the pH optimum for human lysozyme was 5 6. The finding that the complement and lysozyme dependent colilytic activity of human NS had its optimum on the alkaline side of neutrality pH 8.5–9.5 (*Wardlaw* 1962) is difficult to explain. The immunoinactivation of *Toxoplasma gondii* found to be properdin dependent had also an alkaline pH optimum, 8.5–8.7 (*Strannegård* 1967)

The present studies of the effect of added Ca^{++} and Mg^{++} on the NS immobilization reaction revealed that the NS activity inhibited by addition of EDTA could be fully restored and even increased by addition of Mg^{++} but only partly restored if Ca^{++} were added The enhancing effect on the serum activity by Mg^{++} was found to be optimal at a final concentration of approximately 0.005 M. A simultaneous addition of various amounts of Ca^{++} could not further increase the enhancing effect caused by Mg^{++} alone. In fact, the simultaneous addition of Ca^{++} to the serum diluent at concentrations present in NS had even a depressing effect on the serum activity Interpretation of the present results must take into account the various sources of error in this reaction system. Thus, substitution of the phosphate buffers of the medium with other buffers was not compatible with good treponemal survival and accordingly following addition of Ca^{++} to the reaction mixtures, precipitates of calcium phosphate were found (Table 5) Furthermore, addition of Ca^{++} to EDTA treated serum, might bring about a release of Mg^{++} because Ca^{++} has a greater affinity than Mg^{++} to EDTA. This might suggest that complement is activated through the alternate pathway bypassing the early acting complement components. Nothing but Mg^{++} appears to

be involved in this alternate complement activation (*Götze & Müller Eberhard* 1971 *Sandberg & Osler* 1971 *Marcus et al.* 1971)

The optimum Mg^{++} concentration for NS immobilization of treponemes, 0.005 M, was high as compared with the corresponding value, 0.0005 M found to be optimal for complement haemolysis In the latter reaction, Ca^{++} is a necessary co-factor

As regards the bactericidal reaction (*Wardlaw & Pillemer* 1956) to be evoked by NS, the Mg^{++} concentration required for optimal activity ranged around 0.002 M. There was no evidence suggesting that Ca^{++} was necessary for an occurrence of these reactions.

Studies of the effect of added Mg^{++} on the treponemal immobilization by IS are not available in the literature whereas it has been reported that addition of Ca^{++} will consistently increase the immobilizing titres of IS (*Chorpenning & Beers* 1960)

Thus, some differences between the NS and IS immobilization reaction have been found, viz. the rate, the course, and possibly also the ionic strength dependence of the two reactions. The cause of these differences may be due to different types of antibodies participating in the two reactions. There is much evidence that the antibody in NS in accordance with other findings concerning most of the studied "natural" antibodies (*Michael & Rosen* 1963 *Svehag* 1964) is largely associated with immune globulins of the 19 S type (to be published) Conversely the activity of heated immune serum is found in the 7 S fraction only (*Laurell & Hederstedt* 1958, *Julian et al.* 1969) One possible explanation of this observation may be that the SH-compounds of the Nelson medium (Table 1) like 2 mercaptoethanol (*Deutsch & Morton* 1957) will reduce the IgM molecules.

A comparison of the present results concerning the optimal conditions and kinetics of the NS immobilization reaction with the corresponding data concerning other NS reactions suggests that there is good correlation with the bactericidal (*Davies & Wedgewood* 1965) and bacteriolytic reactions (*Wardlaw* 1962)

In spite of the thorough standardization of the test procedures worked out in the present investigation, the titres of one NS tested on different days varied considerably A comparison between titres of different NS tested on different days suggests that the results should be correlated with the titre of a standard serum. The poor reproducibility may be due to structural variations of the treponemal surface as well as to variations in the unavoidable coexisting rabbit tissues, if the latter is derived from different rabbits. The reproducibility of NS titres, determined in experiments performed in one and the same day however was of about the same degree as that for the conventional TPI test (*Hederstedt* 1961) the standard deviation being 0.024 This suggests that the present method will be satisfactory in the further investigations planned.

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GLUCOKINASE AND GLUCOSE 6-PHOSPHATE DEHYDROGENASE IN *NEISSERIA*

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Glucokinase and glucose 6-phosphate dehydrogenase activities are found in species of "true neisseriae" also in the non-saccharolytic ones. The enzymes are not found in the "false neisseriae" (*N catarrhalis N ovis* and *N caviae*) The stimulating effect of glucose on the synthesis of these enzymes, and the electrophoretic behaviour of glucose 6-phosphate dehydrogenase are compared in the different species.

Enzymatic activities corresponding to glucokinase (ATP D-glucose phosphotransferase, E.C. 2.7 1.2) and glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate NADP oxidoreductase, E.C. 1 1 1 49) have previously been found in extracts from *Neisseria meningitidis* (9) The activity of glucokinase was found to be stimulated in cells which had been grown with glucose as the only carbon and energy source.

The present investigation has been performed to study the activities of these enzymes in different *Neisseria* species.

MATERIALS AND METHODS

The *Neisseria* strains, media and extraction procedure were the same as those used in previous investigations (6 8)

Enzyme assays. Enzyme assays were performed as described by *Jyssum et al.* (9) The reaction mixture for the assay of glucokinase contained $MgCl_2$ 5 μmoles, ATP 2 μmoles, D-glucose 20 μmoles, NADP 0.25 μmoles, extract 0.05–0.3 ml, containing 15–25 mg protein per ml, and Tris/HCl buffer pH 8.0 110 μmoles in a total volume of 2.5 ml. Reduction of NADP was followed spectrophotometrically as described (8) Glucokinase activity is expressed as nmoles NADP reduced per mg protein during the first minute of incubation. Usually no exogenous G-6-P dehydrogenase was added to the system. However in *N flavescens* the glucokinase was difficult to demonstrate by means of the endogenous G-6-P dehydrogenase. The "false neisseriae" had no detectable G-6-P dehydrogenase activity When these species were tested for glucokinase activity 0.1 ml extract from *N meningitidis* B 8152/66 (containing 2.1 mg protein) was added. *N meningitidis* B 8152/66 does not possess any glucokinase activity but the G-6-P dehydrogenase is present (10 Table 1)

For the assay of G-6-P dehydrogenase the reaction mixture contained G-6-P 10 μmoles, NADP 0.25 μmoles, extract 0.05–0.3 ml and Tris/HCl buffer pH 8.0 120 μmoles in a total volume of 2.5 ml. Enzyme activity is expressed as nmoles NADP reduced per minute per mg protein.

Agar gel electrophoresis was performed as described (8)

Chemicals. G-6-P was obtained from Sigma Chemical Company St. Louis, Mo., U.S.A. ATP was purchased from Koch-Light Laboratories, Ltd., Colnbrook, Buckinghamshire, England. Other chemicals were those used previously (8)

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Abbreviations: G-6-P: D-glucose 6-phosphate.

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TABLE 1 (continued)

Species	Strain	Glucokinase	G-6-P dehydrogenase
N ovis	199/55	0*	0
	37/59	0*	0
	917/60	0*	0
N caviae	ATCC 14659	0*	0
	NCTC 10293	0*	0

Glucokinase activities are expressed as nmoles NADP reduced during the first minute of incubation per mg protein, G-6-P dehydrogenase activities as nmoles NADP reduced per min per mg protein.
* tested in the presence of extract from *N meningitidis* B 8152/66.
§ nmoles NADP reduced during the first 2 minutes of incubation.

RESULTS

Enzyme activities All strains of "true neisserias" showed significant activities corresponding to glucokinase and G-6-P dehydrogenase, except the non-saccharolytic *N meningitidis* strain B 8152/66, which lacked glucokinase activity as demonstrated previously (10) In the "false neisserias" neither of these enzymes could be detected (Table 1)

Effect of glucose on enzyme activity Extracts were made from cells grown on KC-medium (*N meningitidis*) and Mueller Hinton medium (other *Neisseria*) supplemented with various concentrations of glucose In *N meningitidis* both glucokinase and G-6-P dehydrogenase activities were increased in cells which had been grown on glucose-containing medium. In *N gonorrhoeae N sicca* and *N lactamica* the G-6-P dehydrogenase activity was enhanced by increasing glucose concentration. So was the glucokinase activity at lower concentrations of glucose, but it decreased at higher concentrations (Fig 1) In *N flavescens* and *N elongata* no effect of glucose was seen.

The G-6-P dehydrogenase in *N meningitidis* B 8152/66 was not affected by glucose. This strain and strain M 6 were also grown on solid and liquid KC-medium without additions, and supplemented with 10 mM G-6-P In no instance increased G-6-P dehydrogenase activity was found. In strain M 6 G-6-P also failed to induce increased glucokinase activity Cyclic AMP had no effect on the induction of enzyme activities.

Agar gel electrophoresis of G-6-P dehydrogenase The result of the agar gel electrophoresis is shown diagrammatically in Fig 2. The G-6-P dehydrogenase of all strains tested migrated towards the anode.

DISCUSSION

In all "true neisserias" glucokinase and G-6-P dehydrogenase were found, even in the non-saccharolytic species (*N flavescens N cinerea* and *N elongata*) *N flavescens* has been found to attack some carbohydrates, although inconstantly and is able to produce a polysaccharide from sucrose (1) so it might not be unexpected to find the above mentioned enzyme activities in this species. All known isolates of *N cinerea* and *N elongata* however are indifferent to carbohydrates (1 2 3) What rôle the two enzymes play in the metabolism of these microbes is not known, although one may speculate that they take part in biosynthetic pathways.

The glucokinase has been assayed by means of the endogenous G-6-P dehydrogenase. In *N meningitidis* it has been shown that G-6-P dehydrogenase is the most active of the two enzymes, and that the glucokinase is the rate limiting factor in the assay (9) In the present study only the G-6-P dehydro-

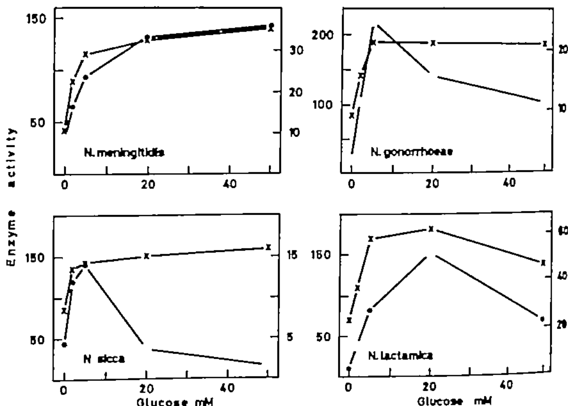


Fig 1 Influence of the glucose concentration in the medium upon glucokinase and G-6-P dehydrogenase activities in *N meningitidis* M 6 *N gonorrhoeae* 1 a *N sicca* CN and *N lactamica* 1379. Left ordinates. G-6-P dehydrogenase (×–×) Right ordinates Glucokinase (●–●) Enzymatic activities are expressed as explained under *Materials and Methods.*

genase of *N flavescens* had too low activity to permit the assay of glucokinase, but this difficulty was avoided by adding extract from *N meningitidis* B 8152/66 to the reaction mixture.

Glucokinase and G-6-P dehydrogenase activities were not found in the "false neisserias" Glucokinase could not be demonstrated even in the presence of extract from *N meningitidis* B 8152/66.

In *E. coli* the G-6-P dehydrogenase is shown to be constitutive (5) Both glucokinase and G-6-P dehydrogenase appear to be constitutive in *Neisseria*. In the saccharolytic species, however the activities of these enzymes is increased in cells grown on glucose-containing media. No enhancement of G-6-P dehydrogenase activity took place in *N meningitidis* B 8152/66, which lacks glucokinase it might then be possible that G-6-P could act as inducer This compound failed to have any effect in the strains B 8152/66 and M 6 in the latter neither the glucokinase was stimulated. However G-6-P is metabolized more slowly than glucose in strain M 6 (9) The relatively low ability of G-6-P to enter the metabolic pathways of the cells, compared to that of glucose might explain why this compound has no effect on the G-6-P dehydrogenase Other compounds might as well be responsible for the induction, but this point has not been further explored. Also the activities of the glutamate dehydrogenases, which were altered by glucose, were not affected by G-6-P (8)

In *N gonorrhoeae* *N sicca* and *N lactamica* glucokinase is stimulated by small concentrations of glucose less stimulated or even

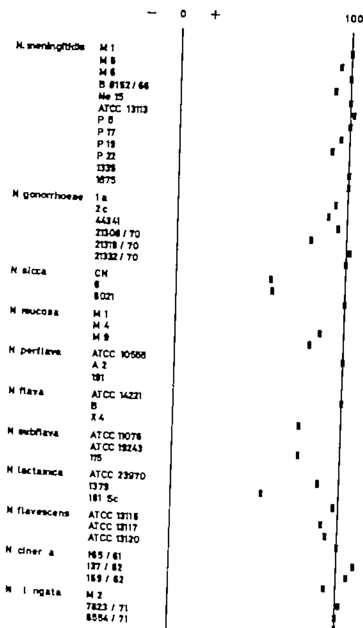


Fig. 2. Electrophoretic pattern of G-6-P dehydrogenase from *Neisseria*, diagrammatically. 0 denotes the application point. The migration distances are given relative to that of G-6-P dehydrogenase from *N. meningitidis* M 6. The anode is to the right.

inhibited by higher concentrations. It is possible that the induction may be masked by catabolite repression by glucose at high concentrations of this compound.

There was considerable variation in the electrophoretic mobility of G-6-P dehydrogenase, even within one species. No pattern could be seen that might contribute to the classification of these bacteria, in contrast to the results obtained by electrophoresis of the glutamate dehydrogenases (6). The same was noted by electrophoresis of G-6-P dehydro-

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6-PHOSPHOGLUCONATE DEHYDROGENASE AND ENZYMES OF THE ENTNER-DOUDOROFF PATHWAY IN *NEISSERIA*

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6-phosphogluconate dehydrogenase, 6-phosphogluconate dehydrase and 2-keto-3-deoxy-6-phosphogluconate aldolase were studied in several *Neisseria* species. 6-phosphogluconate dehydrogenase and 2-keto-3-deoxy-6-phosphogluconate aldolase were present in all strains of "true neisseriae". The synthesis of 6-phosphogluconate dehydrogenase was stimulated by glucose in the saccharolytic *Neisseriae* and its activity was stimulated by NADP in *N. meningitidis* and *N. gonorrhoeae*. 6-phosphogluconate dehydrase was present in most strains of "true neisseriae" except in *N. cinerea* and *N. elongata*. This enzyme was induced by glucose in the saccharolytic *Neisseriae*. In *N. flavescens* its synthesis was inhibited by glucose.

Neisseria meningitidis has been shown to be able to metabolize glucose via the oxidative pentose phosphate and the Entner Doudoroff pathways (8). Activity corresponding to 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase, E.C. 1.1.1.44) was demonstrated, and it was also shown that 6-PG was converted to pyruvate and glyceraldehyde 3-phosphate. The 6-PG dehydrogenase was found to be dependent on the concentration of NADP.

The present investigation was undertaken to compare the 6-PG dehydrogenase in different *Neisseria* species, and to examine the enzymes participating in the Entner Doudoroff pathway: 6-PG dehydrase (6-phospho-D-gluconate hydro-lyase, E.C. 4.2.1.12) and 2 keto-3-deoxy-6-phosphogluconate aldolase (6-phospho-2 keto-3-deoxy-D-gluconate D-glyceraldehyde-3-phosphate lyase, E.C. 4.1.2.14).

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MATERIALS AND METHODS

The *Neisseria* strains, media and extraction procedures were the same as those used in previous investigations (4, 6).

Enzyme assays. 6-PG dehydrogenase was assayed as described by *Jyssum & Jyssum* (8). The reaction mixture contained in a total volume of 2.5 ml $MgSO_4$ 10 μmoles, K_3AsO_4 20 μmoles, NH_2OH 20 μmoles, KCN 5 μmoles, 6-PG 10 μmoles, NADP 1 μmole, extract 0.5 ml, containing 15–25 mg protein per ml, and Tris/HCl buffer pH 7.4 92.5 μmoles. The reduction of NADP was followed spectrophotometrically as described (6). Enzyme activity is expressed as nmoles NADP reduced per minute per mg protein.

6-PG dehydrase was assayed by a slight modifi-

Abbreviations: 6-PG 6-phospho-D-gluconate, KDPG: 6-phospho-2-keto-3-deoxy-D-gluconate.

TABLE 1 *Activities of 6-PG Dehydrogenase 6-PG Dehydrase and KDPG Aldolase in Neisseria*

Species	Strain	6-PG dehydrogenase	6-PG dehydrase	KDPG aldolase
N meningitidis	M 1	6.6	153.9	134.5
	M 5	28.6	89.5	74.9
	M 6	5.6	70.1	162.0
	B 8152/66	12.0	30 7	121.2
	Ne 15	11.0	124.5	88.7
	ATCC 13113	0 4	143.3	152.1
	P 5	14.3	66.2	170.2
	P 17	11 6	291 4	67.9
	P 19	22.6	29.8	103 7
	P 22	15.2	66.9	89.7
	1555	19 4	219 7	102.3
	1875	19.5	57.9	108.6
N gonorrhoeae	1 a	12.9	185.3	175.4
	2 c	9.8	112.8	140.2
	44341	5 0	68.9	229 4
	21308/70	21.2	132.6	111 4
	21319/70	13 2	232.5	191.0
	21332/70	1.3	186.4	198.4
N sicca	CN	27.3	490.5	82 8
	6	45.5	684.0	89.8
	8021	51 4	1982.3	84.7
N mucosa	M 1	30.8	98.6	119.1
	M 4	55.4	907 4	49.0
	M 9	56.4	640 6	64.1
N perflava	ATCC 10555	2.4	452.4	138.4
	A 2	55.2	242.9	148.9
	191	28.7	46.4	145 6
N flava	ATCC 14221	41 1	56.5	178.9
	B	8.0	119 7	189 8
	X 4	3.8	66 8	163.9
N subflava	ATCC 11076	27 1	510.1	128.3
	ATCC 19243	47.3	98.0	107.7
	115	40.1	67 1	71 4
N lactamica	ATCC 23970	9.3	165 9	134.2
	1379	0.9	265.2	279 6
	161 Sc	10.3	281.5	169 1
N flavescens	ATCC 13115	1.8	1537.0	225.2
	ATCC 13117	0.5	284 4	147.5
	ATCC 13120	3.8	389 7	144.2
N cinerea	165/61	9 0	0	84 8
	137/62	24.4	0	103.3
	159/62	23 8	0	118.6
N elongata	M 2	22 5	0	117.2
	7823/71	19.2	0	98 1
	8554/71	20.0	0	200.1

TABLE 1 (continued)

Species	Strain	6-PG dehydrogenase	6-PG dehydrase	KDPG aldolase
N catarrhalis	ATCC 8176	0	–‡	0
	Ne 11	0	–	0
	15016/62	0	–	0
N ovis	199/55	0	–	0
	37/59	0	–	0
	917/60	0	–	0
N caviae	ATCC 14659	0	–	0
	NCTC 10293	0	–	0

Activities of 6-PG dehydrogenase are expressed as nmoles NADP reduced per minute per mg extract protein, of 6-PG dehydrase and KDPG aldolase as nmoles pyruvate formed in 30 minutes per mg extract protein.

Extracts made from cells grown on Heart Infusion agar containing 20 mM glucose.

‡ Not done, see text.

cation of the procedure followed by *Jyssum & Jyssum* (8) in a mixture containing K_3AsO_4 2 µmoles, CH_3COONa 2 µmoles, 6-PG 2 µmoles, extract 50 µl and Tris/HCl buffer pH 7.4 11 µmoles in volume of 250 µl. After 30 minutes of incubation at 37 C the reaction was stopped by adding 250 µl 0.6 M $HClO_4$. After neutralization with 1 M K_2HPO_4 aliquots were assayed for pyruvate by means of lactate dehydrogenase. Enzyme activity is expressed as nmoles pyruvate formed in 30 minutes per mg protein.

KDPG aldolase was assayed in the same way except that 0.2 µmoles of KDPG was used as substrate

Chemicals KDPG was prepared from sodium pyruvate and glyceraldehyde 3-phosphate by extract from *Pseudomonas fluorescens* CCEB 488 (kindly supplied by Dr *K. Kersters* State University Gent, Belgium) according to the method of *Meloche & Wood* (11) 6-PG sodium pyruvate, glyceraldehyde 3-phosphate diethyl acetal and lactate dehydrogenase were purchased from Sigma Chemical Company St. Louis, Mo. U.S.A. Other chemicals were those used previously (6)

RESULTS

6-PG dehydrogenase Activity corresponding to this enzyme was found in all strains of the "true neisserias" (Table 1) In all "false neisserias" this enzyme was absent. In *N meningitidis* and *N gonorrhoeae* enzyme activity was enhanced by increasing concentration of NADP in the assay mixture (Fig 1) Only a slight effect of NADP was found in *N lactamica* and *N elongata* while the enzyme activity in *N sicca* was indifferent to NADP concentration. In *N flavescens* a slight inhibitory effect of high NADP concentration was noted.

Extracts from cells grown on glucose-containing media had higher 6-PG dehydrogenase activity than from cells grown without glucose in 4 saccharolytic species tested (Fig 2) No effect of glucose was found in the non-saccharolytic *N flavescens* and *N elongata*.

6-PG dehydrase and KDPG aldolase KDPG aldolase was demonstrated in all strains of "true neisserias" So was also the 6-PG dehydrase, except in *N cinerea* and *N elongata*. As the KDPG aldolase was absent in the "false neisserias" activity of the 6-PG dehydrase was not detected in these species. The demonstration of the latter enzyme is dependent on the former one in the experimental procedure used.

When tested with different glucose concentrations, the KDPG aldolase was not affected. The activity of 6-PG dehydrase increased with increasing medium concentration of glucose in *N meningitidis* *N gonorrhoeae* and *N lactamica* (Fig. 3) In *N meningitidis* no activity was found in cells grown

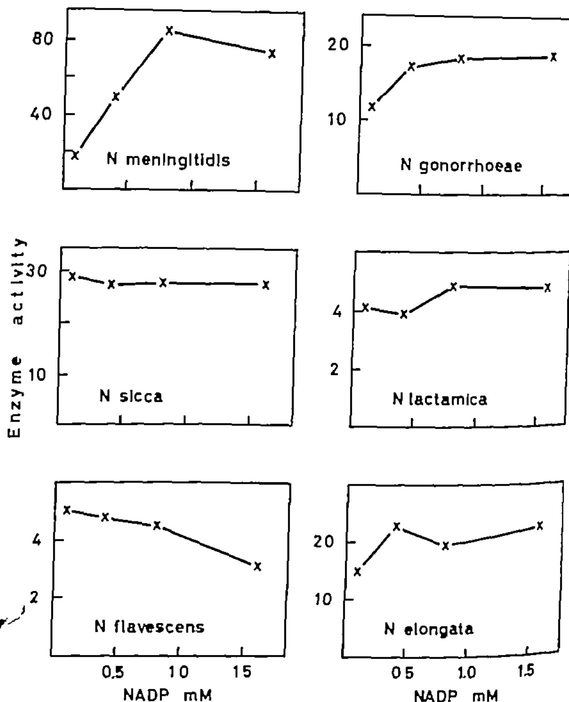


Fig 1 Activities of 6-PG dehydrogenase from *N meningitidis* M 6 *N gonorrhoeae* 1 a, *N sicca* CX, *N lactamica* 1379 *N flavescens* ATCC 13120 and *N elongata* M 2 at different concentrations of NADP Enzymatic activities are expressed as nmoles NADP reduced per minute per mg protein.

without glucose and in *N gonorrhoeae* and *N lactamica* only traces of activities were found.

In *N flavescens* the reaction to glucose was the reverse of that found in *N meningitidis*, high activity was found in cells grown without glucose and decreasing activity with increasing glucose concentration.

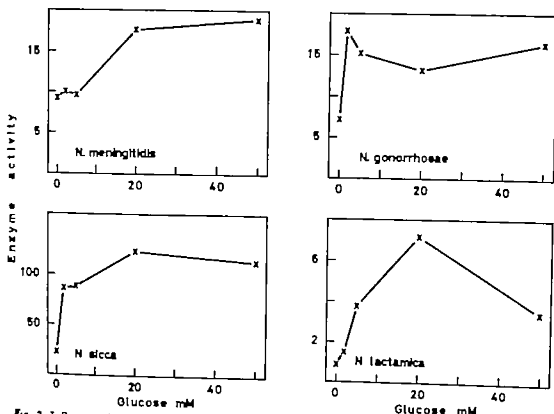


Fig. 2. Influence of the glucose concentration in the medium upon 6-PG dehydrogenase from *N. meningitidis* M 6, *N. gonorrhoeae* 1 a, *N. sicca* CN and *N. lactamic* 1379. Enzymatic activities are expressed as nmoles NADP reduced per minute per mg protein.

In *N. perflava* ATCC 10555 6-PG dehydrogenase activity was demonstrated in cells grown on Heart Infusion agar supplemented with 20 mM glucose, not in cells from blood agar or Mueller Hinton medium with 20 mM glucose. Also the activity of 6-PG dehydrase was dependent on the medium. In *N. perflava* ATCC 10555 enzyme activity was absent in cells grown on blood agar it was absent. In all these strains activity 20 mM glucose, in *N. sicca* CN it could not be demonstrated in cells grown on Mueller Hinton medium with different glucose concentrations, and in *N. mucosa* M 1, *N. flava* ATCC 14221 and *N. flava* B grown on blood agar it was absent. In all these strains activity could be demonstrated in cells grown on Heart Infusion agar with 20 mM glucose.

The demonstration of 6-PG dehydrase is dependent on KDPG aldolase in the present system. One would hence expect the observed activity of the former enzyme always to be lower than that of the latter one. In some strains however the dehydrase seemed to be more active than the aldolase. This difference in enzyme activities might be due to different substrate concentration, as 2 μmoles of 6-PG and 0.2 μmoles of KDPG were used in the assays.

DISCUSSION

Like the G-6-P dehydrogenase (5) 6-PG dehydrogenase is present in all strains of "true neisserias" examined, and is not found in any of the "false neisserias". Also, in the saccharolytic *Neisseria* species the enzyme is more active in cells adapted to glucose, whereas it

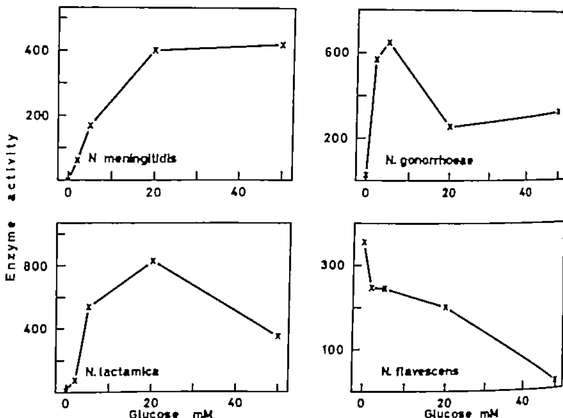


Fig 3 Influence of the glucose concentration in the medium upon 6-PG dehydrase from *N. meningitidis* M 6 *N. gonorrhoeae* 1 a, *N. lactamica* 1379 and *N. flavescens* ATCC 13120 Enzymatic activities are expressed as nmoles pyruvate formed in 30 min tes per mg protein.

is not affected in the non-saccharolytic ones. The enzyme is probably constitutive, as in *Escherichia coli* (3) as its activity is rather high also in cells grown without glucose.

There seems to be some difference between the 6-PG dehydrogenase in the pathogenic species *N. meningitidis* and *N. gonorrhoeae* and that in the other *Neisseria*. In the former species the enzyme is stimulated by a high concentration of NADP while in the latter ones this effect is absent or slight. High NADP concentration may even inhibit enzyme activity How fundamental this difference may be is not known, but it probably involves some difference in the enzyme structure in the different *Neisseria* species.

The Entner Doudoroff pathway has previously been found in *N. meningitidis* (7 8) As the 2 necessary enzymes are present in the saccharolytic *Neisseriae* also *Neisseria* other than *N. meningitidis* would be expected to have an operating Entner Doudoroff pathway This pathway would probably be actually functioning only in cells adapted to glucose as the 6-PG dehydrase is inducible. In *E. coli* 6-PG dehydrase is induced in gluconate-grown cells, not in cells grown in glucose-containing medium (1) The inducer is 6-PG (10) Glucose cannot induce the enzyme probably glucose is not metabolized via the Entner Doudoroff pathway in this species (12) In *N. meningitidis* between 67 and 87 per cent of the glucose is metabolized via this route (7) The nature of the inducer in *Neisseria* is not known. It might be 6-PG as in *E. coli*. This compound must be readily formed from glucose in *N. meningitidis* as glucose is metabolized primarily via the Entner Doudoroff and pentose phosphate pathways (7)

KDPG aldolase seems to be constitutive in *Neisseria* as it was not affected by glucose In

E. coli the activity of this enzyme was increased in cells grown in glucuronate-containing medium (2). It has been suggested that in this species the KDPG aldolase is induced by KDPG and that the high basal level of this enzyme might be attributed to synthesis of KDPG from glyceraldehyde 3-phosphate and pyruvate (2). This might also be the case in *Neisseria*.

In *N. flavescens* the 6-PG dehydrase was active in cells grown without glucose, its activity decreased with increasing glucose concentration, being almost totally inhibited at 50 mM. The apparently constitutive nature of this enzyme in *N. flavescens* strongly contrasts the findings in the saccharolytic *Neisseria*. One cannot exclude the possibility that an inducer is present within the cells, and that its concentration is altered in cells growing on glucose-containing medium or its activity masked by catabolite repression by glucose.

Why the synthesis of 6-PG dehydrogenase and 6-PG dehydrase was so completely dependent on the medium in certain strains of *N. mucosa*, *N. perflava*, *N. flava* and *N. sicca* has not been investigated further. External inducer was present in all media, as the blood agar contained 4.8 mM glucose. Probably some factors in the media have inhibited enzyme synthesis.

No 6-PG dehydrase activity was found in *N. elongata* or *N. cinerea*. Extracts were made from blood agar and from Heart Infusion agar containing 20 mM glucose. One cannot exclude the possibility that enzyme synthesis could take place in cells grown on other media, but the results strongly suggest that the enzyme is absent. The presence of KDPG aldolase in these species is in accordance with the findings of *Kersters* & *De Ley* (9) who in an investigation of 142 strains belonging to 37 genera noted that KDPG aldolase could be present in the absence of 6-PG dehydrase, but not *vice versa*.

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INFLUENCE OF DIFFERENT FACTORS ON THE PRODUCTION OF L-PHASE VARIANTS IN INDIVIDUAL *E. COLI* STRAINS

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L-colony production in 19 E. coli strains was studied. Several factors were found to influence productivity and type of L-growth, e.g. type and concentration of antibiotic, pH and hypertonicity. Individual E. coli strains were found to have different requirements.

Lederberg & *St. Clair* studied the influence of different factors such as pH, sucrose and the concentration of penicillin on the production of L-colonies of *E. coli* in pour plates (7). *E. coli* has a great strain variability as regards L-phase production and the reproducibility appears to vary from strain to strain (7, 2, 1).

It is known that certain *E. coli* strains require higher concentrations of penicillin than the usual 1000 IU/ml in order to produce L-phase growth (7, 10). The concentration of penicillin has been reported to be one of the most critical factors for L-phase production in *E. coli* (7, 10), but also other factors might be responsible for the strain-differences.

The present study was undertaken to obtain some information about differences between *E. coli* strains regarding the requirements for certain factors, such as type and concentration of antibiotic used for induction, pH and the presence of sucrose in the medium.

L-phase variants are cell wall defective bacteria capable of reproduction. On suitable solid media they form colonies known as L-colonies (5). In the present paper we will refer to this classical type of L-colony which has resemblance to the "fried egg"-like colonies of mycoplasma, as "typical" L-colonies. The L-phase growth which is lacking the dense central area of the colony will be referred to as "homogenous" L-colonies. Neither type of colony contained bacillary forms. According to our experience with *E. coli*, the former has ability to become stable and to grow in the absence of penicillin, while the latter has not such ability.

MATERIALS AND METHODS

E. coli strains. The 19 *E. coli* strains studied were the test strains of *E. coli* O-antigen O1–O20, with the exception of strain O14, from the WHO International Escherichia Centre, Statens Seruminstitut, Copenhagen, Denmark.

Culture media. The solid medium used in this study, the TY-medium, has been described earlier (4, 11). The osmolality of the medium was 443 milliosmol/kg. In some experiments, the medium was used without the addition of sucrose (osmolality 546 milliosmol/kg). Further, the medium was used with different combinations of pH and diffe-

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rent concentrations of penicillin, ampicillin and cephalothin. Penicillin G (Benzylpenicillin, Kabi, Stockholm, Sweden) was used in the following concentrations 1000 10,000 and 50,000 IU/ml. Ampicillin (Pentrexyl, Bristol) was used in the concentrations 50, 500 and 5000 mcg/ml, and cephalothin (Keflin, Lilly) in the concentrations 50 500 and 5000 mcg/ml. The pH was adjusted to pH 5.0 6.0 and 7.0 with 5M HCl or 5M NaOH.

Induction of L-phase variants The *E. coli* strains were grown in liquid TY-medium (without agar) in shaken cultures for 5 hours in a waterbath at 37° C, and 0.2 ml was spread over each plate. The plates were incubated aerobically in a 5 per cent CO -atmosphere for 5 days. Parallel plates were incubated anaerobically in certain experiments. When subcultivations of L-colonies were made, the agar-to-agar technique was used (6) The plates were inspected after 5 days for macroscopic growth and by an inverted microscope (125×)

RESULTS

The 19 *E. coli* strains were inoculated onto plates with the three different concentrations of penicillin, ampicillin and cephalothin at pH 5 6, and 7 i.e. a total of 27 combinations.

All strains except strain O7 produced L-phase growth. Five strains produced L-colonies of the "classical" type in at least three of the 27 combinations O2, O8, O15 O17 and O18. In addition, these five strains produced "homogenous L-colonies on the same plate. The "homogenous" type of L-colony was produced by all strains except O7 In most of the strains, the "homogenous" type of colony appeared over a wide range of pH and type and concentration of antibiotic. The "classical" type, on the other hand, had more specific requirements.

Type and concentration of antibiotic The production of "classical" L-colonies at the different concentrations of the antibiotics used is shown in Table 1 The concentration of penicillin G was more decisive than the concentration of ampicillin or cephalothin.

The highest productivity of the "homogenous" L-colonies was found at the lower concentrations of penicillin and ampicillin, whereas the productivity was markedly lower at all concentrations of cephalothin. Four strains, O4 O10, O19 and O20 were totally non-productive with cephalothin. Among all strains, strain O12 had the most abundant production of "homogenous" L-colonies. After one serial transfer to the same type of medium, however the growth changed to the "classical type of L-colonies. Such changes in colonial morphology were sometimes also seen in other strains, such as the strain O2.

Effect of pH on L-colony production. Different strains were found to have their maximum L-phase productivity at different pH (Table 1) Strains O2 and O17 were the only strains which were favoured by low pH. All the other strains had an optimum at pH 6 or 7 The table shows the optimal pH for production of the "classical" type of colony but for most strains "homogenous" L-colonies were favoured by the same pH, except, sometimes the strains O2 and O17

Sucrose E coli (strains O2, O8, O15 O17 and O18) was inoculated onto plates with

TABLE 1 *Concentrations of Different Antibiotics Inducing "Classical" L-colonies in Five E. coli Strains at Optimal pH*

Strain	Optimal pH	Penicillin G* IU/ml	Ampicillin† mcg/ml	Cephalothin† mcg/ml
O2	5–6	1000	500 5000	—
O8	7	1000, 10,000	50, 500 5000	50 500
O15	6–7	1000	50, 500 5000	—
O17	5–6	1000	500 5000	500
O18	6	1000	50 500	50 500

* tested on medium with and without sucrose.
† tested on sucrose-containing medium only

and without sucrose containing 1000 IU penicillin/ml or 500 mcg ampicillin/ml at pH 5 6 and 7 i.e. a total of 12 combinations. The *E coli* strains chosen showed high productivity of L-colonies and produced colonies of the "classical" type.

These strains, except strain O18 produced L-colonies of the "classical" *type both in the* presence and absence of sucrose. Colonies of the "homogenous" type were produced by all of the five strains.

At pH 5 and in the absence of sucrose the strains O8 and O17 had a markedly increased tendency to develop L-colonies of the "classical" type compared to the results obtained on plates containing sucrose. In many experiments, colonies of the "classical" type appeared at pH 5 only in the absence of sucrose. Inversely at pH 6 the "classical" type of L-colony often appeared only on sucrose containing plates. Cross-wise transfers of L-colonies from these induction plates to plates with and without sucrose at pH 5 and 6 respectively confirmed these results. Further strain O8 which did not produce "classical" L-colonies at pH 5 in the presence of sucrose did so if sucrose was withdrawn from the medium. Strain O8 produced large numbers of "classical" L-colonies at pH 6 and 7 both in the presence and absence of sucrose.

Aerobic versus anaerobic incubation. The 19 *E. coli* strains were also incubated anaerobically (pH 7 1000 IU penicillin/ml, plates containing sucrose) Using anaerobic incubation, classical bacterial colonies were absent *or few compared to aerobic incubation, and* consequently the majority of the colonies consisted of L-colonies. The morphology of L-colonies was sometimes influenced by anaerobic conditions. Strain O12 produced L-colonies of the "classical" type during anaerobic induction, but in CO_2-supplemented air the colonies were of the "homogenous" type.

Bacterial colonies As mentioned above bacterial colonies were few or absent on penicillin-containing plates incubated anaerobically Using aerobic incubation however bacterial growth was abundant with 1000 IU penicillin/ml, which was also the most effective concentration for L-colony production. 12 of the present 19 *E coli* strains showed bacterial growth under these conditions. In the same experiment only 3 of the strains showed bacterial growth on plates containing 50 mcg ampicillin/ml but not at higher concentrations. Using 50 mcg/ml cephalothin, *17 strains produced bacterial colonies and* using 500 mcg/ml, 11 strains produced bacterial colonies.

DISCUSSION

The present study has confirmed previous observations on the strain variability in *E coli* as regards L-colony production (2,7). Different *E coli* strains may require different concentrations of penicillin for production of the L-phase (7 10) However the present study has also shown that *E. coli* strains are selectively favoured as regards L-colony production, by changes in cultural conditions other than changes in penicillin concentration.

The present results confirm the statement by *Schumann & Taubeneck* that L-colonies of *E coli* in contrast to *Proteus* cannot be divided into strictly separable groups on the basis of colonial morphology (10) Some of the present strains produced two morphologically different types of L-colonies, either alone or concomitantly In contrast to the authors mentioned we have so far only been able to obtain stable L-phase variants from *E coli* strains which produce the "classical" (or "fried egg-like") type of L-colonies (4, 11)

None of the present *E coli* strains was favoured by higher concentrations of penicillin. As regards production of the "classical" type of L-colony only one strain was productive at 10.000 IU penicillin G/ml. Sharply contrasting to this narrow spectrum for L-colony production by penicillin was the present finding that ampicillin, and to some extent cephalothin, *induced L-phase growth* over a much wider range of concentrations In further contrast to penicillin G bacterial growth was often absent at those concentra-

tions of ampicillin which were most favourable for L-phase production One possible explanation of these findings may be a presence of certain differences in the biochemical mechanism of action of the agents, as has been suggested in the case of penicillins and cephalothin (8) Another explanation is that the acid-labile penicillin G is degraded faster than the acid-stable ampicillin by acid metabolic products from the growing microorganisms. The production of spheroplasts (and thus probably also L-phase variants) from Gram-negative bacilli by penicillins has been shown to occur at concentrations well above those which inhibit growth (7 9) and it is possible that the concentration necessary for spheroplast formation is maintained for a longer period of time in the case of ampicillin than in the case of penicillin G

The presence of sucrose in the medium has been reported by others to be indispensable for L-phase induction in *E coli* (7 10) The present TY medium is hypertonic even in the absence of sucrose, and thus might explain why sucrose was not found necessary However the type of medium can influence the requirement for sucrose by *E coli* L-phase variants, as shown by *Lederberg* & *St Clair* who found that a small number of *E. coli* L-colonies developed in casein-digest meat agar but not in nutrient agar (7)

Two *E. coli* strains (O2 and O17) produced abundant growth of "classical" L-colonies on plates without sucrose in the presence of 1000 IU penicillin at pH 5 but not, or very sparsely at pH 6 and pH 7 whereas no "classical" L-colonies were found on the parallel sucrose-containing plate at pH 5 but abundantly at pH 6. These differences are not fully understood. As regards one *E. coli* strain, a similar phenomenon was observed by *Gnarpe* & *Edebo* (3) on plates with low osmolality A possible explanation of the results obtained with plates without sucrose might be that, in acid environment, the cell membrane and/or cell wall of the L-phase variants is stabilized, similarly to findings by these authors in the case of *E. coli* spheroplasts in liquid media (3) Such an explanation might well be valid also in the case of the present hypertonic TY-medium. However the present strains which grew as L-colonies at pH 5 on plates without sucrose but not on the parallel plate with sucrose have been found either to have a lower pH optimum than the other strains (strains O2 and O17) or to have an extremely high capacity for L-phase transformation (strain O8) Since changes in pH might be more pronounced in the sucrose-containing medium, possibly resulting in an environment too acid for growth, this might account for the absence of "classical" L-phase growth on sucrose containing medium with initial pH 5

The different ability of individual *E coli* strains to produce L-phase growth is well-known (2, 7 1 10) The present results suggests that individual *E coli* strains also have different requirements as regards the combination of external factors necessary for L-phase production.

The skilful technical assistance of Mrs. *Ingrid Wergeløg* is gratefully appreciated.

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^{125}I LABELLING OF *CANDIDA ALBICANS* BY ELECTROLYSIS

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The aim of this investigation was to establish an easy method for radiolabelling of large quantities of heat-killed *Candida albicans*. The yeast particles were to be used in studies of phagocytosis in human monocytes cultured *in vitro*. The results show that it is possible to label quantities of $3–4 \times 10^9$ Candida particles in one single run by electrolysis of a Na^{125}I solution. The surface antigens of the Candida were found to be intact after the electrolytic iodination process.

Elemental iodine will react with proteins on the cell surface, and probably also with lipids in the cell membrane and within the cell. Elemental iodine is set free at the anode by electrolysis of a Na^{125}I solution (1). To economize with the radioactive iodine, non-radioactive NaI was added in small, but sufficient amounts for the electrolysing process. It was found to be convenient to operate with volumes of 500 ml per run. The factors affecting the electrolytic process, as the Candida concentration, the amount of Na^{125}I, the ratio non-radioactive/radioactive iodine and the current were tested. A method by which to obtain a reasonable degree of labelling of *Candida albicans* will be reported.

MATERIALS AND METHODS

A 700 ml closed Quickfit glass equipment was prepared (Fig. 1). At one of the top entries (A) an anode of polished platinum foil (35 cm^2) was fixed. At the other entry (C) the cathode (a thread of platinum) was fixed and separated from the anode zone by means of a dialyser membrane connected to a glass tube. The cathode compartment was filled with 0.9 per cent NaCl solution. *Candida albicans* heat-killed at 60° C for two hours, were suspended in a solution of Na^{125}I (code IAR, Kjeller, Norway), NaI and 0.9 per cent NaCl in sterile water and introduced into the anode compartment. The whole equipment was placed on an electromagnetic stirrer (Cenco pyro-mag stir.5). The current intensity delivered from a transformer was kept constant and controlled by an amperemeter.

Samples of Candida could be taken during the process from the top entry marked S (Fig. 1). These samples were washed several times in large volumes of saline. The radioactivity in the final sediment containing Candida and in the supernatant was measured in an automatic Wallac GM crystal gamma radiation counter. The numbers of yeast particles in the suspensions were measured in an electronic particle counter (Coulter model Fn).

In order to test the labelling stability the Candida were suspended in Parker 199 tissue culture medium (Flow Ltd) with 20 per cent human AB serum, after being washed 5 times in large volumes of saline. The concentration of the yeast particles was adjusted to 1.5×10^6 per ml, and volumes of 2.5 ml were added to plastic Petri dishes, 50 mm diameter (Nunclon, Nunc A/S, Denmark). The dishes were incubated at 37° C in an atmosphere containing 5 per cent CO$_2$ in air with a humidity of 100 per cent. Samples were collected after different periods of incubation, and the radioactivity in the cell free portion of the medium and in the sediment containing Candida particles was measured after centrifugation.

After being washed 5 times in large volumes of saline, the radiolabelled heat-killed *Candida al-*

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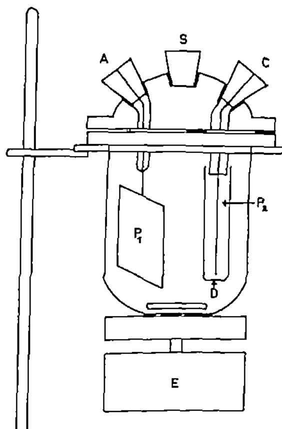


Fig 1 Equipment used in the electrolytic iodination process. A Anode entry C Cathode entry S Sample collecting entry P_1 Polished platinum electrode P_2: Platinum thread electrode D Dialyser membrane, E Electromagnetic stirrer device.

bicans were suspended in saline dispensed in small volumes and stored at − 20° C.

Rabbit antiserum against *Candida albicans* was prepared as follows: A suspension of *Candida albicans* in saline was injected into the ear veins of two rabbits in increasing volumes from 0.25 to 2 ml per injection. Six injections were given in the course of three weeks. In the two first injections, heat-killed *Candida albicans* were used. In the last four injections, living Candida were used. At the end of the fourth week the rabbit sera were found to contain antibodies agglutinating *Candida albicans* in a 1/128 dilution.

RESULTS

A convenient degree of labelling was obtained by using a 500 ml saline suspension of heat killed *Candida albicans* containing 7.5×10^6 yeast particles per ml, and with 35 mg NaI

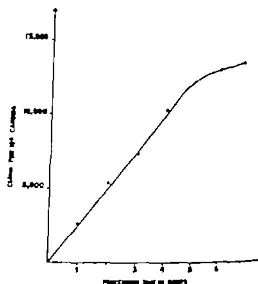


Fig 2 The diagram shows the yield of labelling expressed as c.p.m. per 10^6 Candida measured in samples collected at different times of the radiolabelling process. Heat-killed Candida albicans were suspended in a solution containing 35 mg NaI, 1 mCi ^{131}I (0.1 mg Na^{131}I) and 0.9 per cent NaCl in sterile water. The Candida concentration was 7.5×10^6 per ml. This solution was electrolyzed at a constant current of 5 mA.

and 1 mCi Na^{131}I (approximately 0.1 mg Na^{131}I) added. This suspension had to be electrolyzed for about 7 hours at a constant current of 5 mA. The specific activity of the Candida labelled as mentioned as a function of the processing time is shown in Fig. 2. The yield of this process was found to be 15 600 counts per minute (ct/min)/10^6 Candida, i.e.

TABLE 1 *The Influence of Variations in the Ratio Radioactive/Non-radioactive Iodine on the Yield of the Labelling Process.*

The ratio NaI/Na ^{131}I in mg/mg	Ct/min per 10^6 Candida
3500/1	975
700/1	14200
350/1	16400

The figures listed are the final yield of labelling from 3 different experiments where different ratios between NaI and Na ^{131}I were used. The other factors concerning the iodination process were kept constant in the 3 experiments.

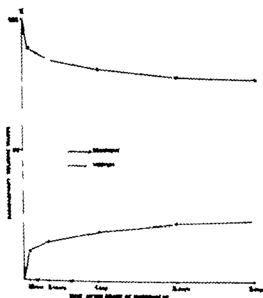


Fig. 3. ^{125}I-labelled heat-killed *Candida albicans* suspended in complete culture medium and incubated at 37 C. Samples containing 3.5×10^{8} radiolabelled Candida in 2.5 ml complete medium were collected after different periods of incubation and the radioactivity in the sediment containing Candida particles and in the cell free medium was registrated after centrifugation.

about 75 Candida particles per ct/min. The relative degree of labelling was found to be about 12 per cent of the radioactivity added.

If all the above mentioned factors were scaled down to 1/10 the final result was found to be about 18 600 ct/min per 10^{8} Candida, or a slight increase in the degree of labelling.

An increase of the Candida concentration was found to inhibit the process of electrolysis probably by coating the dialyser membrane. With a Candida concentration of 20×10^{8} per ml, the whole process stopped after one hour with a poor result of 250 ct/min per 10^{8} Candida.

Variations in the ratio radioactive/non-radioactive iodine were found to influence the result of the labelling process. Table 1 shows that the specific activity of the radioiodine must be high to obtain an acceptable degree of labelling.

If the current was increased above 5 mA, the suspension was found to turn yellow as a sign of production of more free I than the cells were able to pick up The free I_2 can react with O_2 and water and produce acids which are not able to label the cells Thus, an increase in the current intensity in order to shorten the time of the process was found to be of no advantage

After washing 5 times in large volumes of saline only about 1 per cent of the activity was found in the supernatant after the last centrifugation.

The labelled Candida were stored deep-frozen suspended in saline and after 1–2 months the radioactivity was found to be reduced according to the half life of ^{125}I Radiolabelled *Candida albicans* stored at – 20 C thus showed a good stability

If incubated in complete culture medium at 37 C, the loss of radioactivity during the first 15 min was found to be about 10 per cent, with an increase to about 20 per cent after 2–3 days (Fig. 3)

Radiolabelled and non labelled heat killed *Candida albicans* were found to be agglutinated to the same degree by rabbit antibodies against *Candida albicans*

DISCUSSION

The iodination method presented above gives an acceptable radioactivity per Candida without any sign of destructive effect on the cells. The surface antigens of the radiolabelled Candida were found to be intact. By this method it is possible to label large quantities of Candida in a relatively easy way The labelling appeared to be fairly stable during storage and washing in saline. If incubated in complete culture medium at 37 C, the release of radioactivity to the medium was found to be moderate. An increase in the amount of ^{125}I added, an increase in the time of electrolysis combined with a lowering of the current intensity (1) a reduction of the concentration of Candida in the suspension and processing at a smaller scale are all

factors which can improve the yield of labelling, if wanted or needed.

Advice and technical assistance from the Institute of Marine Biochemistry, the University of Trondheim are gratefully acknowledged. The heat-killed *Candida albicans* were kindly supplied by the Department of Microbiology, Trondheim Central Hospital.

This work was supported by grants from the *Norwegian Cancer Society* and from the *Norwegian Research Council for Science and the Humanities*.

The author is a fellow of the Norwegian Research Council for Science and the Humanities.

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STRUCTURAL AND FUNCTIONAL PROPERTIES OF BLOOD MONOCYTES CULTURED *IN VITRO*

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Mononuclear phagocytes from human venous blood were cultured in plastic Petri dishes and on glass coverslips in Petri dishes. After an initial cell loss, the cell cultures showed a good structural and functional stability. No signs of cell proliferation were noticed. Young cultured cells had an appearance like that of monocytes while older cultured cells appeared like macrophages with the ability to engulf and digest heat-killed *Candida albicans*. The young cells showed the ability of engulfing Candida, but were unable to break down the engulfed corpuscles.

The blood monocyte is a circulating mononuclear leucocyte that comprises 3–7 per cent of the total numbers of blood leucocytes. *In vivo* studies using labelling have shown that these cells originate in the bone marrow from promonocytes (3, 7). The monocytes leave the circulation and develop into tissue macrophages. In studies of mice (3 6 7) the monocytic origin of tissue macrophages, e.g. peritoneal macrophages, Kupffer cells in the liver alveolar and skin macrophages, has been shown by labelling.

As tissue macrophages, the mononuclear phagocytes serve as scavenger cells in chronic inflammatory reactions.

The morphological and functional properties of the lymphocyte have been well documented. We know in some detail the ability of the lymphocyte to differentiate into an antibody-producing plasma cell or into an aggressive lymphocyte with reactive properties towards the stimulating antigen.

Monocytes and macrophages are also of importance in the immunological reaction towards antigens. Human lymphocytes from sensitized individuals transform *in vitro* into blasts in the presence of the sensitizing antigen. The blastoid trasformation, with an increase in DNA synthesis, was reduced by the removal of adhesive monocytes (4). There are also indications that the macrophage can serve as a non-specific effector cell in the immune response. Macrophage function appears to be modified by components produced by antigen-stimulated lymphocytes (5). These components, called lymphokines, induce increased phagocytic and bactericidal activity in the macrophages.

The aim of the present work was to standardize a culture technique for blood monocytes and to study the morphology and function of such cells cultured *in vitro*.

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MATERIALS AND METHODS

Separation Techniques

Venous blood from healthy adults was defibrinated in Erlenmeyer flasks using a glass stirrer. Two

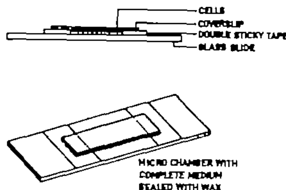


Fig. 1 The figure shows the micro chamber made of a glass slide with two ribbons of double sticky tape streched across the slide. The distance between the two ribbons of tape is approximately 2 cm. The depth of the chamber is approximately 800 μm. The medium is drawn into the chamber *after application from a Pasteur pipette along the* open slit between the glass slide and the coverslip. The chamber is sealed with wax all around the coverslip.

different methods for the separation of leucocytes were tested. According to the first method defibrinated blood mixed with Dextran (6 per cent Intradex, Nyco) in the proportion 8 parts of blood to 2 parts of Dextran, was incubated at 37 C for 45 min. During this time, the erythrocytes will sediment while the granulocytes and mononuclear cells remain in the supernatant. The second method for separation was carried out as described by *Boyum* (1967) using Ficoll (Pharmacia, Sweden)/Isopaque (Nyco Norway) in the separation medium.

The cells were washed twice in Hank s balanced salt solution (BSS) and suspended in Parker s medium 199 (TC 199) supplemented with 1-glutamin (1 mg/ml) and 20 per cent human AB serum, obtained after high speed centrifugation. Streptomycin and penicillin were added in concentrations of 100 μg/ml and 100 units/ml, respectively

Cell Cultures

The cell suspensions prepared as described above were cultured in two different ways. In one of the culture variants, plastic Petri dishes (Nunclon) with a diameter of 50 mm were used. The culture volume was 2.5 ml. The cell suspensions to be cultured were applied to the Petri dishes by means of a 5 ml graded pipette. The dishes were placed horizontally in a National CO -incubator at 37 C with 5 per cent CO in air and with 100 per cent humidity In the other culture variant, heat-sterilized coverslips (Leighton type 11 × 35 mm) were used. The glass coverslips were placed in duplicate in plastic dishes of the same type as those used in the other variant. One half ml of the cell suspensions to be cultured was distributed on the individual coverslips.

In both culture variants, the cells were allowed to attach to the plastic or glass coverslip surface during an incubation period of 90 minutes. The medium containing the non-adherent cells was then removed, the cell layer on the bottom of the Petri dishes or the coverslips was flushed with medium to remove loosely attached cells, and 2.5 ml of fresh, complete medium was added to each culture dish. After the first day of incubation the medium was changed, subsequently on every fourth day

Preparation of Candida and Cell Debris

Candida albicans were heat killed at 60° C for 2 h. The yeast particles were added to the cultured cells in a ratio of about 10:1 for the 90 minutes old phagocytes and in a ratio of about 100:1 for the 8 days old phagocytes. Cell debris was prepared from homologous or autologous mononuclear leucocytes suspended in complete medium and frozen and thawed six times.

Technique for Registration of Cell Number and Vitality

The number of cells in the suspensions applied to the Petri dishes and in the medium removed

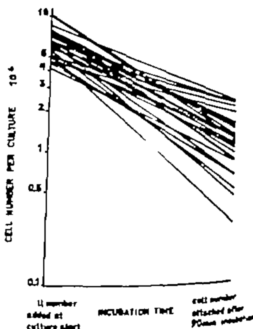


Fig. 2 Mononuclear blood cells adherent to the bottom of culture dishes after an incubation period of 90 minutes. Numbers of added cells and numbers of attached cells in 30 consecutive cultures.

TABLE 1 *Separation of Leucocytes from Defibrinated Human Blood. A Comparison between Two Different Separation Procedures with Regard to the Isolation and Culture of Blood Monocytes*

Leucocyte count per mm³ blood	Separation medium	Leucocyte yield (per cent)	Ratio mononuclear cells/leucocytes	Mononuclear cell yield (per cent)	Adhesive cells per culture dish after 4 days
5000	Ficoll/Isopaque	18	80	15	12000
	Dextran	35	31	11	13800
5000	Ficoll/Isopaque	13	88	14	10800
	Dextran	20	44	9	10200
4500	Ficoll/Isopaque	11	91	10	10200
	Dextran	14	35	5	7200
4200	Ficoll/Isopaque	10	98	9	14400
	Dextran	32	25	8	7800
6300	Ficoll/Isopaque	22	80	17	19300
	Dextran	35	48	18	15700
2500	Ficoll/Isopaque	30	92	28	18700
	Dextran	58	36	21	21700

after incubation for 90 min was counted in an electronic particle counter (Coulter Counter Model Fn).

The cells cultured in Petri dishes without coverslips were observed and counted directly using a Reichert inverted microscope with phase contrast. Coverslip cultures could also be studied directly without removal of the coverslips. Usually however the cells cultured on coverslips were studied only at the time of harvesting. The coverslips were then removed from the Petri dish, rinsed in complete medium and inverted on microscopic glass slides with two ribbons of double, sticky tape (Scotch brand double sticky tape, no. 665) stretched across the slides. In this way the coverslips with the cultured cells were placed like a roof on the top of a small chamber (Fig. 1) which subsequently was filled with culture medium, sealed with wax and studied in a Leitz Laborlux phase contrast microscope with automatic equipment for photographic registration of the cultured cells. The cell number per coverslip was estimated from the number of cells counted in 20 microscopic fields, multiplied with a constant factor. This factor was estimated from the total number of fields per coverslip. The chambers could be incubated at 37 °C for several hours without evidence of evaporation of the medium. The effect on the cells of different components was easily studied morphologically. Phagocytosis of visible corpuscles added to the medium was observed and photographed in the incubation chambers.

The suspended cells were differentiated into mononuclear and polymorphonuclear cells after staining with an acridine orange staining solution. One half volume of the staining solution containing 11.5 mg acridin orange per 100 ml TC 199 pH 7.0 was added to the cell suspensions followed by observation on a Laborlux fluorescence microscope with a ×40 objective and an Osram mercury vapour lamp as the light source. A BG 12 blue

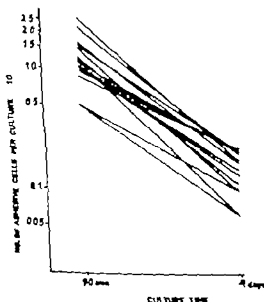


Fig. 2. Survival of human monocytes cultured in plastic Petri dishes. Numbers of attached cells in 15 consecutive cultures after culture for 90 min and 4 days.

exciter filter was used with a yellow-orange absorption filter The number of cells showing a yellow green fluorescence as a sign of cell vitality was determined.

Phagocytosis of Heat Killed Candida

Following the addition of yeast particles incubation was continued for 10 min. The coverslips were then removed rinsed in Hanks BSS inverted on micro chambers and studied by phase contrast microscopy For the digestion studies the micro chambers were incubated and studied by phase contrast microscopy for 24 hours.

RESULTS

Table 1 gives the yields of separated leucocytes, the differential counts in the obtained cell suspensions and the survival of adhesive monocytes. The separation procedure using

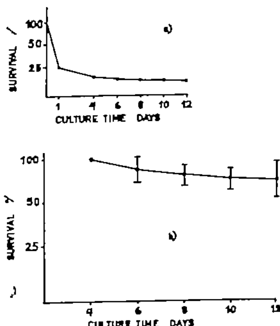


Fig 4 Survival of human monocytes cultured in plastic Petri dishes.

a) Numbers of adhesive mononuclear phagocytes expressed as a percentage of the cell numbers at culture start. Mean values are given from 17 consecutive culture lines with duplicate or triplicate culture dishes harvested on each of the days indicated.

b) Numbers of adhesive mononuclear phagocytes from the fourth to the twelfth day of culturing expressed as a percentage of the cell number on day four Mean values and standard deviations are given

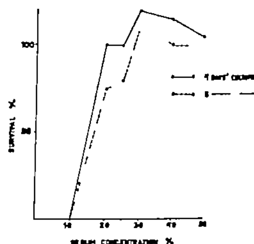
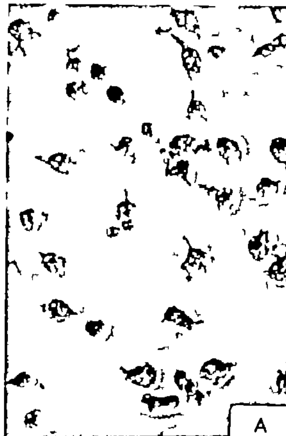


Fig 5 Survival of human monocytes cultured in medium containing different concentrations of human serum. Each mark is the number of adhesive mononuclear phagocytes in each culture dish, expressed as the percentage of the cell number in the culture dish containing medium with 20 per cent serum on day 4

Dextran as an erythrocyte-aggregating agent gave a higher leucocyte yield than Isopaque/Ficoll separation where predominantly mononuclear cells were obtained. The yield of mononuclear cells was about equal in the two procedures. As indicated in Table 1 there was no significant difference in the number of adhesive cells obtained by the two techniques after 4 days culturing.

The vitality of the cells in suspensions and of the adhesive cells varied between 90 and 95 per cent as judged by supravital staining and by phagocytosis of heat killed *Candida albicans*

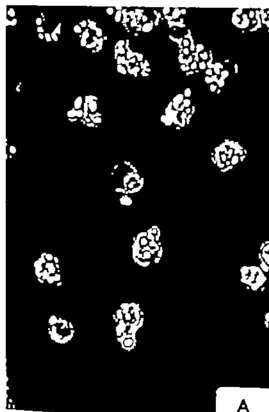
The number of leucocytes to adhere to the bottom of the plastic Petri dishes after an incubation period of 90 min is given in Fig 2 The numbers of adhesive cells were estimated as the difference between the leucocyte count added to the culture dishes and the numbers of cells removed after incubation for 90 minutes. The numbers of adhesive cells from the suspensions obtained by the Ficoll/Isopaque separation technique varied between 5 and 40 per cent of the numbers of mononuclear blood cell added to the culture dishes the mean value of adhesive cells being


A


B


C


D



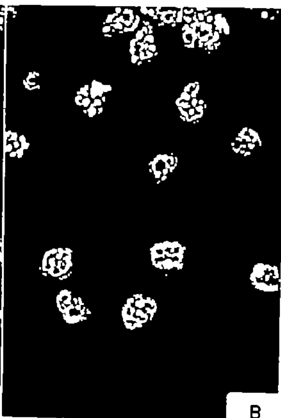


Fig. 6. a) 90-minute-old mononuclear phagocytes with engulfed heat-killed *Candida albicans* incubation for 10 min after addition of yeast particles.
b) The same cells after incubation for one hour. Detachment of the cells from the glass surface has occurred.
c) Phagocytosis of Candida by mononuclear phagocytes after 24 hours incubation. The phagocytic cells have detached from the glass surface and are found at the bottom of the microchambers. Marked cell aggregation is seen. The Candida particles are still visible in the cells. Phase contrast × 1295.

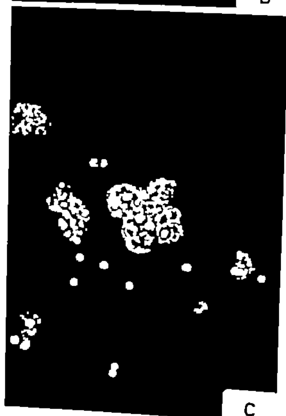


Fig. 7. a) Adhesive mononuclear blood cells after culturing for 90 min. The cell surface shows many small projections. A few lymphocytes can be seen.
b) Adhesive mononuclear phagocytes after 4 days' culturing.
c) An adhesive mononuclear phagocyt cultured on glass for 8 days. The perinuclear cytoplasm is filled with phase-dense granules.
d) A multinuclear giant cell after 10 days culturing. [N]umerous phase-dense granules and phase transparent vacuoles are present in the central cytoplasm. Phase contrast × 1295

ing the inoculation of the dishes. Consideration was also paid to the volume measurement in replicate cultures.

The survival of cells cultured on coverslips was not so easily estimated from direct counts of adherent cells due to less clear light transmission through the plastic and glass structures at the bottom of culture dishes. From counts of cell numbers on coverslips inverted in incubation chambers a rapid fall in numbers of cells from the first to the fourth day of incubation was found followed by relatively stable cultures during the following week, similar to the findings in the cultures containing plast adhesive cells

After culturing for 90 min the adhesive cells had spread over the surface and membrane pseudopodia were seen (Fig 7a) The cells contained no phase-dense granula at this stage of development. A few adhesive lymphocytes could be seen. After 4 days in culture the adhesive mononuclear phagocytes had increased in size and had acquired phase dense granula localized in the perinuclear region (Fig 7b) After 8 days, the phase-dense granula in the cells had increased in number (Fig 7c) Two different morphological types of cells were observed round epithelial-like cells and spindle-shaped, fibroblast like cells (Fig 7b) After 8 days' culturing, scattered multinuclear giant could be observed (Fig 7d)

As shown in Fig 8 cells cultured for 90 min engulfed heat killed Candida in a number of 5 to 10 particles per cell The phagocytic cells tended to round up and detach from the surface after 10 to 15 min. No definite digestion of the engulfed yeast particles was observed.

Fig 9 shows the different steps in the phagocytic process the adherence engulfment and digestion of heat killed Candida in 8-day-old cultures. In the course of one hour the engulfed corpuscles became located in the perinuclear region. After 24 h of incubation, no intact particles could be seen in the cells. In Fig 10 an "overloading effect" with cell damage was observed when Candida had been added to the cultures in excess. Some cells became vacuolated and appeared to be unable to digest the engulfed Candida.

DISCUSSION

The aim of the present work was to study the morphology and function of blood monocytes cultured *in vitro* Both monocytes and granulocytes are adhesive cells with ability to spread on a surface and for phagocytosis. Human monocytes can be cultured for at least one month whereas granulocytes will degenerate after a few days' culturing

The above described separation techniques are short and simple procedures for the isolation of leucocytes from human blood. Cell vitality as judged by cell survival and phagocytosis did not significantly differ in cultures with cells obtained by the two separation techniques. The main advantage of the separation procedure using Ficoll/Isopaque for culturing blood monocytes is the low contamination of granulocytes and erythrocytes. This enables a better morphological and functional study of the mononuclear phagocytes at culture start.

A high proportion of the adhesive cells lost their spreading ability and became detached from the plastic or glass surface during the first days of incubation. An average contamination of five per cent granulocytes was found using the Ficoll/Isopaque separation technique Thus the granulocytic contamination alone cannot explain the great cell loss. Short time adhesion of lymphocytes may be

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Fig 9 Digestion of heat-killed *Candida albicans* in 8-day-old human mononuclear phagocytes during an incubation period of 24 h (approximately 10 yeast particles added per cell)

a) Yeast particles adherent to the cell surface after incubation with the yeast for 10 minutes.

b) and c) Phagocytic cells after incubation for two and four hours, respectively. The yeast particles are engulfed and are located in the central cytoplasm.

d) After 24 hours incubation no intact particles can be seen. The cytopla m h granular ap- pearance Thread-lik projections of d [illegible] membrane can be seen. Phase contrast × 1445


A


B


C


D

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Fig 9 Digestion of heat-killed *Candida* adherent in 8-day-old human mononuclear phagocytes during an incubation period of 24 h (approximately 100 yeast particles added per cell)

a) Yeast particles adherent to the cell surface after incubation with the yeast for 10 minutes.
b) and c) Phagocytic cells after incubation for two and four hours, respectively The yeast particles are engulfed and are located in the central cytoplasm.
d) After 24 hours incubation no intact particles can be seen. The cytoplasm is granular in appearance. Thread-like projections of the cell membrane can be seen Phase contrast X 1255.

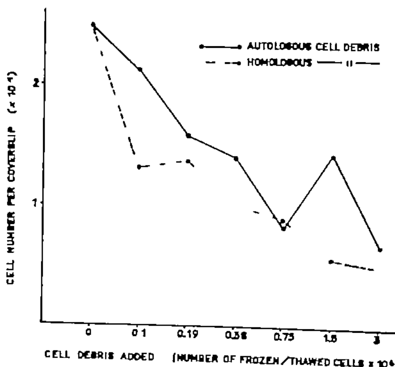


Fig. 11 Phagocytosis of cell debris. Influence of cell debris on the survival of mononuclear phagocytes cultured on glass coverslips. Two series of cultures incubated in the presence of different numbers of frozen/thawed autologous or homologous cells. 4 days culture time.

of importance and in fact, some of the adhesive cells found after incubation for 90 min were small mononuclear cells. Crowding effect with insufficient nutrition and surface available for spreading may be of importance. A higher proportion of adhesive cells was found if the initial cell number was low

Phagocytosis and cellular adhesion are both functions which depend on a vital cell membrane Therefore, phagocytosis and cell adhesion may interfere with each other An experiment was therefore undertaken to see whether phagocytosis of cell debris could contribute to the extensive cell loss. As shown in Fig 11 the culture dishes containing the highest amount of cell debris had a reduced cell number after 4 days culturing The cells in these cultures showed morphological changes indicating intracellular digestion. In cultures containing a high number of dying and fragmented cells, phagocytosis of cell debris may thus cause detachment of many adhesive phagocytes which themselves in turn become fragmented and cause detachment of other phagocytes. A vicious circle may in this way lead to the destruction of the monolayer culture.

The detachment of adhesive phagocytic cells from plastic or glass surface during the phagocytic process may not necessarily be an expression of cell damage. The phagocytic cells appeared to round up concomitant with the phagocytic process. They may probably regain the ability to attach to the surface.

Following the initial cell loss the adhesive cells showed a good stability with only slight reduction in cell numbers during several days' culturing.

A remarkable observation was the change in cell size and cytoplasmatic structure which was observed during the first week of culturing (Fig. 7) It is known that mononuclear blood cells leave the circulation, for example in inflammatory reactions, and develop into tissue macrophages. *Ebert & Florey* (2) found that blood monocytes could undergo

such a transformation. By implanting cover slips subcutaneously they found that blood cells adhered to the coverslips and that some of the cells acquired structural appearance like macrophages as seen in tissue preparations.

As shown, blood monocytes cultured *in vitro* reflect the same structural changes. Concomitant with this morphological alteration, a change was also observed in the cell function. The phagocytic ability increased and the cells became capable to digest the engulfed Candida corpuscles, an ability which was not found in mononuclear phagocytes after a short culture period.

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PHAGOCYTOSIS OF HEAT-KILLED RADIOLABELLED *CANDIDA ALBICANS* BY HUMAN BLOOD MONOCYTES CULTURED *IN VITRO*

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In vivo experiments indicate that blood monocytes differentiate into macrophages after leaving the circulation (2, 3 4) Monocytes separated from venous blood and attached to glass surfaces undergo a similar differentiation *in vitro* (8) In this report, the phagocytic function of monocytes at various stages of this differentiation is demonstrated by means of a technique using radiolabelled *Candida albicans* With a view to attachment and engulfment as well as to digestion of yeast particles, quantitative changes of the phagocytic function were found during the first 8 days of culturing.

The human monocyte can be cultured *in vitro* for at least four weeks. During the first eight days of culturing the cells mature into macrophages. This differentiation comprises both morphological and functional changes (8) The results from an investigation which was carried out to support our previous findings by quantitation of phagocytosis, will be reported here

For this purpose, an *in vitro* model using monocytes cultured on coverslips and radiolabelled *Candida albicans* was established. The following factors which seem to influence the phagocytic process were tested in the culture system

a. The age *in vitro* of the cultured cells.
b. The number of cells per coverslip.
c. The number of Candida added.
d. The time available for attachment and engulfment.
e. The time of the digestion phase of phagocytosis.

Other factors like pH, addition of medium, incubation conditions and washing procedures were kept constant.

The results obtained by this culture model show clearly an increase in the phagocytic ability of the cells during the first eight days in culture.

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MATERIALS AND METHODS

Monocytes were separated from venous blood obtained from healthy blood donors. Mononuclear leucocytes were separated from other blood cells by centrifugation in a Ficoll (Pharmacia, Sweden)/Isopaque (Nyco Norway) medium as described by *Bøyum* (1) After washing, the mononuclear cells were suspended in Parker 199 tissue culture medium (Flow Ltd) with 20 per cent human homologous AB-serum and dispensed in 0.5 ml olumes to glass coverslips (Leighton type 11 × 35 mm) (8) Glass-adhesive cells were estimated to be monocytes and non-attached cells to be lymphocytes.

Candida albicans heat-killed at 60 C for 2 hours were labelled with ^{125}I by means of electrolysis (5 6)

Test Model 1

Coverslip cultures after different periods of culturing (90 min- 1 day 4 days- 8 days) were tested for phagocytic ability following the addition of a constant number of Candida and harvested after a period of phagocytosis varying from 3¾ min to 1 hour The coverslips were washed 3 times in Hanks balanced salt solution (BBS) and the radioactivity on the coverslips registered in a Wallac GM gamma radiation counter Cell numbers on the coverslips were counted in a Leitz Laborlux microscope with phase contrast by means of a micro chamber technique (8) The cell numbers in the Candida and leucocyte suspensions were counted in an electronic particle counter (Coulter Model Fn)

Test Model 2

Cell suspensions containing different cell concentrations were added to the coverslips and cultured for 90 min 1 day 4 days, and 8 days. A constant number of Candida was added to each coverslip and the culturing continued for 10 min before harvesting. The coverslips were washed and recorded as mentioned above

Test Model 3

Different amounts of Candida were added to coverslip cultures 90 min 1 day 4 days, and 8 days after culture start All cultures were incubated for a further 10 min before harvesting. The coverslips were washed and registered as described above.

Test Model 4

A constant number of Candida was added to coverslip cultures after culture periods of 90 min, 1 day 4 days and 8 days After a further incubation for 10 min the coverslips were picked up washed 3 times in Hanks BSS and placed in a new Petri dish containing fresh culture medium. The cultures were harvested after a further incubation period of 15 min 3 hours 1 2 and 3 days. The cover-

TABLE 1 *The Influence of the Length of the Period of Phagocytosis on th Phagocytic Capacity and the Adherence of Human Monocytes Cultured on Coverslips*

Age in vitro of the cultured cells	Time of incubation with Candida in min	Cell number per coverslip × 10		Ct/min per coverslip × 10^3	
		A	B	A	B
90 min	3¾	240	289	37*	58*
	7½	267	206	59	85
	15	202	169	75	103
	30	227	139	61	108
	60	168	83	63	96
1 day	3¾	109	106	22	19
	7½	143	150	42	44
	15	187	88	56	63
	30	134	83	49	64
	60	147	56	62	59
4 days	3¾	69	74	8	13
	7½	59	44	18	22
	15	53	42	26	24
	30	53	19	25	10
	60	65	14	36	12
8 days	3¾	82	137	27	94
	7½	87	89	50	145
	15	80	59	56	137
	30	83	68	54	136
	60	84	68	68	199

* The figures listed are the means of duplicate registrations in four experiments performed at different stages in the development of macrophages from human blood monocytes. The experiments were performed as described as test model 1 A Added 2.5 × 10^6 Candida per coverslip, a medium degree of saturation, B Added 5.0 × 10^6 Candida per coverslip which gives a rather high saturation of the cells with Candida particles.

slips were registered microscopically for cell number and radiologically for amounts of Candida left. The medium from each Petri dish was collected centrifuged at 2000 g for 5 min, and the radioactivity in the supernatant and the sediment was measured separately

RESULTS

In monocyte cultures tested for periods of from 90 min up to 4 days after culture start, the kinetics of the attachment/engulfment phase of phagocytosis was found to be rather equal (Table 1 Fig 1–2) In 8 days old cells, however a more rapid uptake of Candida was registered, especially if a rather high number of Candida had been added to the cells (Fig. 1–2)

After phagocytosis of a smaller number of Candida, only a slight degree of cell detachment was found in cells aged 90 min up to 4 days while 8 days old cells were not

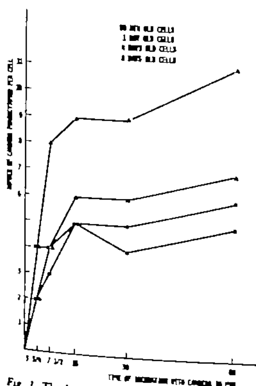


Fig 1 The kinetics of the attachment/engulfment phase of phagocytosis t different times fter culture start 2.5 × 10⁶ Candida particles were added per coverslip (medium saturation)

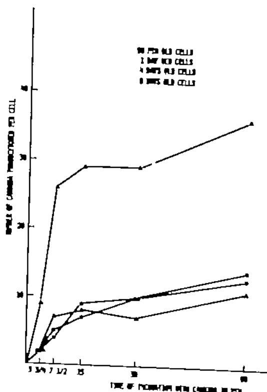


Fig 2 The kinetics of the first steps of phagocytosis when a high dose of 5.0 × 10⁶ Candida was added to the cells.

found to detach at all (Fig 3) After attachment/engulfment of a high number of yeast particles, the cells showed a greater tendency to detach from the coverslips (Fig. 4) Cells cultured for 90 min up to 1 day were found to detach rather slowly while 4 to 8 days old cells detached more rapidly

Fig 5 shows that in 90-min-old cells the estimated number of Candida engulfed per cell decreased with an increase in cell number per coverslip This relationship does not seem to be linear At cell numbers above 90 × 10³ cells per coverslip, the number of engulfed Candida per cell was found to be almost constant. At lower cell concentrations the cells were found to engulf more Candida. The results obtained from cells cultured for 4 and 8 days before testing showed the same trend.

The estimated number of Candida engulfed per cell increased with an increase in the number of Candida added (Table 2, Fig 6) This relationship was found to be

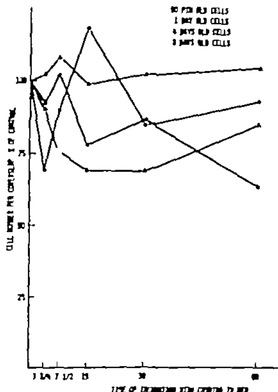


Fig 3 Cell numbers listed in Table 1 as relative values to show a comparison between the ability of the cells of different ages to remain on the glass surface during the process of phagocytosis. 2.5×10^{6} Candida were added per coverslip. Cell cultures without Candida added served as controls.

at low and medium Candida concentrations. At high concentrations, however a level of saturation was apparent, indicating a maximal phagocytic capacity under the given conditions (Fig. 6) In cells aged 90 min up to 4 days this level was found to be approximately 10 Candida engulfed per cell, while 8 days old cells were able to engulf more than 20 Candida per cell. The tendency of cells to detach from the glass surface during phagocytosis was found to increase with increased amounts of Candida added (Table 2)

Registration of the digestion phase of phagocytosis showed that monocytes cultured for 90 min were almost unable to digest Candida (Fig 8) The ability of the cells to digest Candida was quantified as the percentage of the added radioactivity found in a cell free portion of the medium covering one cover slip In controls without cells, 4 per cent of the radioactivity was found to be released to the medium after 15 min, 6 per cent after 3 hours and approximately 12 per cent after 1–3 days (Fig 7) This passive release was *not corrected for in the values listed in Fig* 8–12 and must be taken into consideration when the results are discussed. After 1 day of digestion, the release from cells aged 90 minutes and transfer to the medium was 26 per cent Only 2 per cent of the radioactivity was left on the coverslips, and microscopical examination revealed only a few scattered cells with undigested Candida. The high percentage of the radioactivity found in the sediment reflects undigested Candida in detached cells, verified microscopically After 2 days of digestion, the distribution of the radioactivity was found to be similar Micro-

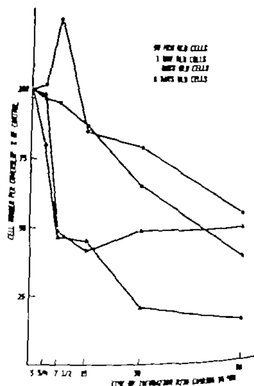


Fig. 4 The kinetics of the detachment of monocytes cultured on coverslips after addition of a high number of 5.0×10^{6} yeast particles. Cell numbers are given as percentages of the numbers in control cultures without Candida added. Compare fig 3.

TABLE 2. The Influence of the Number of Candida Added on the Phagocytic Capacity of Cultured Monocytes and the Ability of the Cells to Remain Adherent to the Coverslips

Age in vitro of the cultured cells	Number of Candida added per coverslip $\times 10^{6}$	Cell number per coverslip at harvesting $\times 10^{3}$	Ct/min per coverslip $\times 10^{3}$
90 min	0.33	138	10*
	0.7	151	18
	1.4	164	34
	2.75	87	55
	5.5	115	83
	11.0	76	85
1 day	0.4	107	11
	0.75	59	11
	1.5	77	26
	3.0	94	36
	6.0	45	45
	12.0	74	80
4 days	0.35	49	10
	0.7	77	18
	1.4	44	23
	2.75	60	35
	5.5	48	45
	11.0	53	67
8 days	0.35	20	9
	0.7	16	11
	1.4	19	24
	2.75	16	24
	5.5	25	52
	11.0	16	44

The figures listed are the means of triplicate registrations in four experiments, performed as described in test model 3

especially the cells showed signs of lysis and death, both in the sediments and on the coverslips.

Monocytes cultured for 1 day had an improved ability to digest Candida compared to cells cultured for 90 min before start of the test (Fig. 9). The amount of released radioactivity transferred to the medium was found to be at the same level as that for 90-min-old cells after 1 day of digestion. However after 4 days of digestion, the release was found to be 64 per cent, indicating a delayed but improved ability to digest Candida. During the first day of the experiment, the radioactivity found in the sediment was rather high being 45 per cent after 5 hours. Registrations of the coverslips showed a rapid fall in the values during the first 5 hours. These observations indicate that cells cultured for 1 day before test start also detach from the glass surface, but this detachment is less prominent than in cultures containing 90-min-old cells. At the end of the experiment, the radioactivity in the sediment was found to be about 20 per cent, indicating either a tendency of the cells to re-attach to the glass surface or a tendency of the detached cells to digest Candida.

Fig. 10 demonstrates the ability to digest Candida after engulfment in monocytes cultured for 4 and 8 days before test start. After 1 day of digestion the release of radioactivity and transfer to the medium was 54 per cent in the case of cells cultured for 4 days, while the release was 74 per cent in the case of 8 days old cells. After 3 days of digestion the difference was insignificant. This indicates that 8 days old monocytes digest Candida more rapidly than monocytes cultured for 4 days. Both types reached the same almost

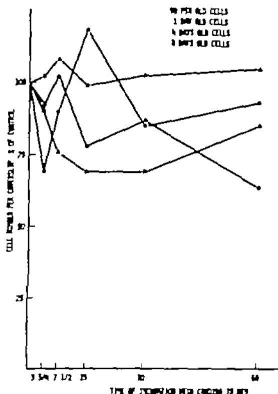


Fig 3 Cell numbers listed in Table 1 as relative values to show a comparison between the ability of the cells of different ages to remain on the glass surface during the process of phagocytosis. 2.5 × 10⁶ Candida were added per coverslip. Cell cultures without Candida added served as controls.

at low and medium Candida concentrations. At high concentrations, however a level of saturation was apparent, indicating a maximal phagocytic capacity under the given conditions (Fig 6) In cells aged 90 min up to 4 days this level was found to be approximately 10 Candida engulfed per cell, while 8 days' old cells were able to engulf more than 20 Candida per cell. The tendency of *cells to detach from the glass surface during* phagocytosis was found to increase with increased amounts of Candida added (Table 2)

Registration of the digestion phase of phagocytosis showed that monocytes cultured for 90 min were almost unable to digest Candida (Fig 8) *The ability of the cells to digest* Candida was quantified as the percentage of the added radioactivity found in a cell free *portion of the medium* covering one coverslip In controls without cells, 4 per cent of the radioactivity was found to be released to the medium after 15 min, 6 per cent after *3 hours and approximately 12 per cent after* 1–3 days (Fig 7) This passive release was not corrected for in the values listed in Fig 8–12 and must be taken into consideration when the results are discussed. After 1 day of digestion, the release from cells aged 90 minutes and transfer to the medium was 26 per cent. *Only 2 per cent of the radioactivity* was left on the coverslips, and microscopical examination revealed only a few scattered cells with undigested Candida. The high percentage of the radioactivity found in the sediment reflects undigested Candida in detached cells, verified microscopically After 2 days of digestion the distribution of the radioactivity was found to be similar Micro-

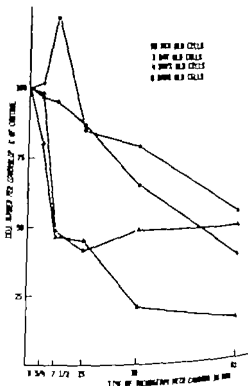


Fig. 4 The kinetics of the detachment of monocytes cultured on coverslips after addition of a high number of 5.0 × 10⁶ yeast particles. Cell numbers are given as percentages of the numbers in control cultures without Candida added. Compare fig 3

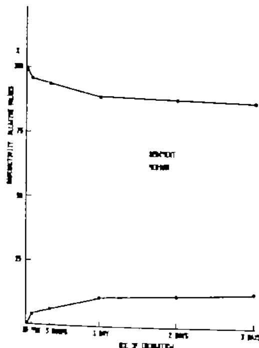


Fig. 7. The passive release of radioactivity from radiolabelled Candida. The Candida were treated as described as test model 4.

cultures showed that the difference between cells cultured for periods of from 90 min up to 4 days was rather small. Both the speed of this first step of phagocytosis and the maximal phagocytic capacity registered was found to be almost at the same level. From the 4th to the 8th day of culturing, however, a more rapid uptake of yeast particles and a two-fold increase in the phagocytic capacity were registered.

After addition of a small number of Candida to the monocyte cultures, only a slight tendency towards cell detachment from the coverslips was noticed in younger cultures while 8 days' old cells remained on the glass surface. After having phagocytosed a large number of Candida, the tendency to detach was found to be more prominent. These observations show that the ability of the cells to attach/engulf large particles as Candida is markedly improved during the first 8 days of culturing. Both attachment/engulfment of particles and attachment to the glass surface are membrane functions of the cell. A greater phagocytic capacity, a more rapid uptake of particles as well as the ability to stay attached to the glass surface are signs of an improved membrane function. If cells were overloaded with Candida, they were found to detach more rapidly. This may reflect that when a large number of large particles are attached/engulfed by the cell, so much of the cell membrane will be involved in this process that it will strongly interfere with the cell adhesiveness to the glass surface. Thus, as compared with younger cells in the same condition, the more rapid detachment shown in cells overloaded with Candida and aged 4 to 8 days, may also reflect an improved membrane function.

The final step of phagocytosis, i.e. digestion of the particles, was also found to be

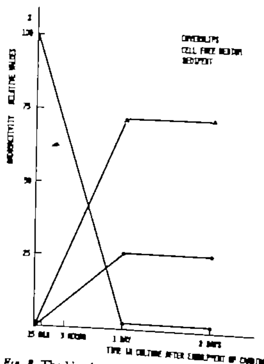


Fig. 8. The kinetics of digestion of Candida by monocytes cultured for 90 min. The experiment was carried out as described as test model 4. A number of 1.5 × 10⁶ Candida was added per coverslip. The figure shows the distribution of the radioactivity in the cell-free medium, the cells on the coverslips, the sediment and the cells on the coverslips at various stages of the digestion process.

highly dependent upon the age *in vitro* of the cultured cells. The results obtained by quantitative measurements show that digestion of yeast particles only occurs in cells cultured for more than 1 day before test start. Furthermore, the digestion capacity was found to be significantly higher in cells cultured for 8 days than in cells aged 1 and 4 days (8).

Consequently the phagocytic ability of blood monocytes cultured *in vitro* improves markedly concomitant with the morphological change to macrophages. *In vivo* a similar development has been suggested. The human blood monocytes originate in the bone marrow (7). After a period of approximately 30 hours, the monocytes leave the circulation and differentiate into tissue macrophages, peritoneal macrophages, alveolar macrophages and Kuppfer cells in the liver (2, 3, 4). The *in vitro* observations therefore immitate the differentiation process taking place *in vivo*.

Several technical and methodological points are of importance. An inverse relationship between the cell numbers and the number of engulfed Candida per cell was found when coverslips containing low cell numbers were tested. As the cell number per coverslip was increased, a point was reached above which a constant number of Candida engulfed per cell was found. In older cell cultures this steady state was found at lower cell numbers than in young cell cultures. The minimal cell number for this steady ability may be related to the cell number necessary to make an even monolayer with cell to cell contact. The relationship between the age *in vitro* of the cultured cells and the minimal cell number for the constant cellular phagocytic ability mentioned above may partly be explained by the fact that the surface of

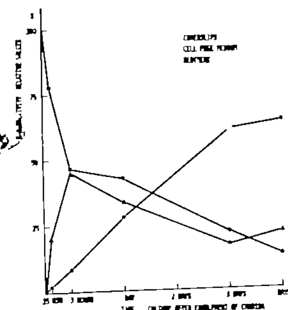


Fig. 9. The kinetics of digestion of Candida by monocytes cultured for 1 day before test start. A number of 1.5 × 10⁴ Candida was added per coverslip. The values given are comparable with the values in Fig. 8.

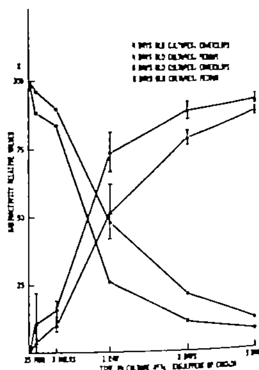


Fig. 10. Comparison between the kinetics of the digestion of Candida by monocytes cultured for 1 and 8 days before test start. The values are the means of the registrations from three experiments in each group ± SD of the means. A number of 1.5 × 10⁴ Candida per coverslip was added, and the cell number per coverslip was at the same level in the 6 experiments. (Approximately 20 Candida per cell were added.)

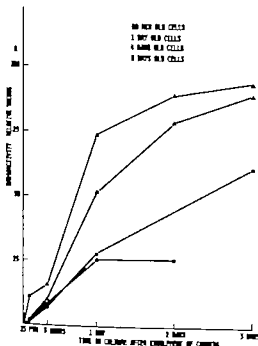


Fig. 11. Comparison between the digestion capacity of monocytes cultured for different times before test start measured as the transfer of released radioactivity to the medium.

cells cultured for 8 days is about 5 times greater than the surface of 90-min-old cells. A possible explanation of the increased number of engulfed Candida per cell found on coverslips containing low cell numbers may be a greater cellular ability to take up Candida when the cells grow separately on the cover slips without cell to cell contact. A greater part of the cell surface will then be available for attachment of particles. Quantitative studies of the influence of different factors on the phagocytic ability of monocytes cultured on coverslips therefore necessitate the use of monolayer culture containing constant cell numbers per coverslip.

The amount of Candida particles engulfed per cell and the tendency of the cells to detach from the glass surface were found to be dependent on the time available for attachment and engulfment of free Candida present in the medium (Table 1). It was therefore considered to be important to use a fixed period of time for the attachment and engulfment stages of phagocytosis. Since the number of Candida engulfed per cell was found to increase with the time available for engulfment, the maximal phagocytic capacity measured by adding excess of Candida is limited by the chosen period of time.

The digestion capacity was found to be dependent upon the number of Candida added in relation to the cell number. With great amounts of Candida added, the cells had to use a longer period of time for the digestion of the yeast particles, though finally reaching the same level as when smaller amounts of Candida were added. In studies of the influence of different factors on the phagocytic process it is of importance to use a constant relationship between the cell number per coverslip and the number of Candida added per coverslip.

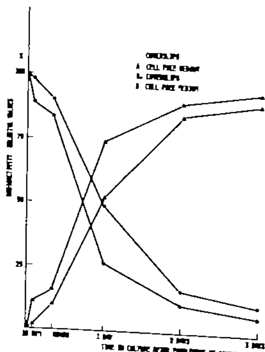


Fig. 12. The influence of the number of Candida added per cell on the kinetics of digestion of Candida by monocytes cultured *in vitro* for 8 days before test start. In one set of experiments about 90 Candida per cell (A) and in the other about 75 Candida per cell (B) were added. The mean values of the results from 3 experiments in each set are given in the figure. The experiments were performed as described as test model 4.

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FAILURE TO DEMONSTRATE IMMUNOGLOBULIN E IN GLOMERULI OF PATIENTS WITH VARIOUS NEPHROPATHIES

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An immunofluorescence study of 119 kidney biopsies from a variety of renal affections, including transplants, is presented. The investigation was carried out with special reference to the possible presence of IgE, but simultaneously other immunoglobulin classes (IgG, IgA, IgM) as well as complement (C3) and fibrinogen were sought for. Out of the 119 biopsies examined, 117 were negative if tested with a monospecific anti-IgE conjugate. The only definitely positive finding of IgE came from two patients with amyloidosis.

The use of the immunofluorescence technique has increased the information to be obtained by examination of kidney biopsies. It has provided evidence that it is very likely that many forms of glomerular disease in man have an immunological mechanism by analogy with experimental models in animals. Such studies have established that the glomerular injury can result from two distinct mechanisms: the deposition of antigen-antibody complexes in glomeruli (immune complex nephritis) or the formation of antibodies to the glomerular basement membrane (anti-GBM-nephritis). Both these phenomena lead to secondary reactions which are supposed to induce the actual damage of the basement membrane (7).

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Both complex nephritis and anti-GBM nephritis have been shown to occur in man. The immunofluorescence technique allows a certain extent of differentiation between these two types of glomerulonephritis. Other forms of glomerular disease can similarly be revealed. The immunoglobulin and complement deposits are granular and discontinuous in immune complex nephritis, being linear and continuous in anti-GBM nephritis (7, 13). Other patterns and distributions of immune deposits have been observed in various other types of kidney diseases (13).

The role of the different immunoglobulins present in glomerular deposits has been discussed. IgG has been found in the majority of cases, but also IgA and IgM have been demonstrated. The presence of IgE in kidney biopsy materials from patients with nephrotic syndrome has recently been reported by *Ger*

ber & Paronetto (8) The main object of this study was to investigate whether their findings could be confirmed At the same time it was possible to study the distribution of other immunoglobulins as well as complement and fibrin in glomeruli and their association with different types of renal diseases. These latter findings will be published and discussed in detail elsewhere

MATERIAL AND METHODS

Microscopic equipment An Orthoplan fluorescence microscope (E. Leitz GmbH Wetzlar Germany) fitted with incident illumination and a HBO 200 mercury vapour arc as light source was used for all examinations. A BG 38/UG 1 combination served as excitation filters together with a KP 490 interference filter A TK 495 dichroic mirror combined with a K 495 barrier filter was selected. A K 510 filter was used as an additional barrier Specimens were viewed with 25/0.65 40/0.75 and 54/0.95 (oil immersion) objectives together with low power oculars (6.3 ×) The total magnification was 160 × 250 × and 340 × respectively

Fluorescent conjugates Sera from rabbits immunized with Fc fragments of human IgE were pooled and fractionated on Sephadex G 200 The IgG fraction was then labelled with fluorescein isothiocyanate (FITC) as described by *Bergquist & Schilling* (1) Protein was determined according to the method described by *Lowry et al.* (12) while the McKinney method (14) was used for fluorescein estimation. The molar F/P was calculated to 2.0 and the protein amount to 0.9 per cent.

Monospecificity was controlled by means of standard particles according to *Bergquist & Kreisler* (2) The conjugate was allowed to react with five different kinds of particles made of IgG IgA IgM, IgE and albumin, respectively A positive reaction was observed only with IgE-particles when different preparations were viewed in the fluorescence microscope Furthermore the same conjugate has been used f r immunofl orescence studies in diseases of the skin. Both in biopsies and in serum from patients with bullous pemphigoid, IgE-antibodies directed against the basal membrane of skin could be found in about 10 per cent of all cases (3) This conjugate was used in dilutions of 1:2 and 1:5

The rest of the conjugates were purchased from different sources which are listed below These conjugates except anti-IgA were unattainable in sufficient quantities from one batch. Therefore different products had to be used from time t time although the brand was always the same.

Purchased conjugates

1 Heterospecific anti-human γ-globulin (National Bacteriological Laboratory Stockholm, Sweden) with molar F/P ratios around 2.5 and antibody concentrations (IgG) of 3.0 mg/ml. The working dilution was 1 10

2 Monospecific anti-human IgA (National Bacteriological Laboratory) with a molar F/P ratio of 2.4 and specific antibody concentration of 0.9 mg/ml. The working dilution was 1:6

3 Anti-human IgG anti-human IgM, anti-human β C (C3) and anti-human fibrinogen were purchased from Hyland Laboratories (Costa Mesa, Ca., USA) These conjugates were used at dilutions of 1:5

Specimen handling Kidney tissue was obtained by percutaneous biopsy and transferred on the same day in saline moistened cotton gauze to the laboratory where it was rapidly frozen with liquid CO_2. Within a maximum of two days, sections of a thickness of 5 µm were cut in a cryostat (W Dittes, Heidelberg Germany) which was operated at −20 C. Sections were subsequently stained in such a way that every biopsy could be examined for the presence of γ-globulin IgG IgA IgM, IgE, C3 and fibrinogen. All reagents were tested before use on normal renal tissue obtained at autopsy No fluorescence was observed with any of the conjugates. Incubation was always carried out at room temperature for 30 min in a moist chamber. Washings were performed in buffered saline of pH 7.4 Specimens were eventually mounted on microscopic slides with buffered glycerol of pH 7.4 and covered with cover slips. The slides were read within one hour after preparation.

Material Over a period of six months, a total of 119 biopsies were examined for the presence of deposits of complement, fibrinogen and immunoglobulins, notably IgE. The nomenclature used for the description of light microscopic findings was principally in accordance with that of Cameron (6) A variety f kidney ailments, including transplants, were studied. The greatest single group comprised patients with chronic glomerulonephritis, among whom some had a rather severe nephrotic syndrome. The second largest group consisted of patients with microscopic haematuria and/or albuminuria but with normal renal function, where light microscopy of biopsy sections had shown so-called minimal changes. Eight biopsies (5 per cent) originated from cases of idiopathic nephrosis (lipoid nephrosis) this term being reserved for patients with nephrotic syndrome and minimal histological changes (4) Five of these patients were treated with steroids. The remaining biopsies were obtained from patients with a variety of kidney diseases (Table 1)

side, results similar to ours have recently been reported by *Roy et al.* (15) and also by *Lewis et al* (11) One possibility which fits an early report by *Boyden* (5) could be that the goat anti-IgE conjugate used by *Gerber & Paronetto* crossreacted with certain cellular structures. Another possibility is that the re actions were due to the presence of heterophilic antibodies reacting with the conjugated goat gamma globulins. Such anti bovine antibodies occur in more than 70 per cent of a population (9)

Two patients with amyloidosis had heavy deposits of amyloid tissue in their glomeruli and IgE was found in these deposits together with other immunoglobulins. These findings, however are of less interest as there is no reason to believe that the localization of amyloid in glomeruli is an immunological phenomenon

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COMPARATIVE STUDIES OF THE PARACORTICAL POST CAPILLARY VENULES OF NORMAL AND NUDE MICE

An Electron Microscopic Study

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The ultrastructure of the post-capillary high endothelial venules in the axillary and inguinal lymph nodes of normal mice and mice which suffer from a congenital aplasia of the thymus (nude mice) was studied. Although the lymph nodes of nude mice showed severe depletion of small lymphocytes in the paracortex and in most parts of the outer cortex the ultrastructure of many post-capillary venules was completely normal. The endothelial cells had a high cytoplasm with a prominent Golgi apparatus, many mitochondria, ribosomes and strands of rough endoplasmatic reticulum. In contrast to normal mice, both lymphocytes and polymorphonuclears traversed the vessel wall of the post-capillary venules, being constantly localized between and not inside the endothelial cells. In the wall of post-capillary venules of the nude mice degenerating leucocytes were often seen to be subjected to phagocytosis by the endothelial cells. A finding—not noticed in previous studies—was the occurrence in both nude and normal mice of an intimate contact between a few of the traversing lymphocytes and the endothelial cells. In areas with such an intimate contact there was a fusion of the glycocalyces of the lymphocyte and endothelial cell membranes. The endothelial cells showed in many cases signs of an intense secretion activity with secretory granula obviously being liberated into the intercellular spaces.

The post-capillary high endothelial venules have aroused a considerable interest in recent years since *Gowans & Knight* (1964) demonstrated the central role of the post-capillary venules in the recirculation of lymphocytes between blood and lymph. Furthermore *Vincent & Gans* (1970) suggested that these vessels are important in the regulation of lymphocyte levels in peripheral blood. In line with this, profound alterations of the post-capillary venules occur in mice and rats which have been made lymphopenic either by neonatal thymectomy or by prolonged drainage of lymphocytes from a thoracic duct fistula (3, 9). Recent ultrastructural studies of post capillary venules of neonatally thymectomized mice however suggest that the vessels only display changes where a coincident heavy lymphocyte depletion is evident in the lymphatic tissues (5).

In order to obtain a further insight into the importance of an intact thymus function to the development of post-capillary venules, the ultrastructure of venules of nude mice has been investigated. As this mouse suffers from a

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congenital aplasia of the thymus (8) its lymphatic tissue displays many of the features characteristic of neonatally thymectomized mice (9 14)

MATERIAL AND METHODS

A stock of outbred NMRI mice carrying the mutation nude (nu/nu-NMRI) and their normal haired littermates (nu/? NMRI) were studied. The animals were kept under very clean conditions and fed sterilized mouse pellets. They were housed in plastic cages and, one week every month, streptomycin was added to the drinking water If treated in this way the nude mice have an average lifespan of about 6 months while the haired littermates live about 2 years. Only healthy two to three months old mice without any signs of infection were studied. Five nu/?-NMRI and five nu/nu-NMRI mice were studied.

Technique for electron microscopy Dissection of the axillary and inguinal lymph nodes was made under ether anaesthesia. Some nodes were immediately fixed in 1 per cent osmium tetroxide buffered with 0 1 M cacodylate (pH 7 4) for 1 hour Other lymph nodes were prefixed in glutaraldehyde 2.5 per cent with 0.1 M cacodylate for 2 hours, followed by fixation for 1 hour in osmium tetroxide. After fixation the specimens were dehydrated in graded series of alcohols and propylene oxide embedded in Epon 812 and sectioned in an LKB ultramicrotome. 400 Å sections were stained with uranyl acetate and lead citrate and examined in a Hitachi Electron Microscope Some of the lymph nodes were fixed *in toto* in 2.5 per cent glutaraldehyde buffered with 0.1 M cacodylate for 24 hours and dehydrated and embedded as mentioned above. These specimens were sectioned at 1 micron and stained with 1 per cent toluidine-blue and examined in the light microscope.

RESULTS

The axillary and inguinal lymph nodes of 2 to 3 months old nude mice are characterized by a severe depletion of small lymphocytes in the paracortex and in most parts of the outer cortex of the nodes (Fig 1) In these lymphocyte depleted areas, completely developed post-capillary venules could be traced from the deep layers of the outer cortex stretching throughout the paracortex (Fig 2)

The ultrastructure of the high endothelial cells from lymph nodes of nude mice does not differ significantly from that of nu/?-controls (compare Figs. 3 and 4) The Golgi apparatus is the most prominent organelle in the cytoplasm of the high endothelial cell together with numerous coated and uncoated vesicles (Fig 5) Moreover mitochondria and accumulations of free ribosomes as well as strands of rough endoplasmatic reticulum are frequent findings. All these organelles have been described previously (2)

Whereas the ultrastructure of the high endothelial cells from normal and nude mice was identical, some differences between the two strains of mice were encountered concerning the leucocytes which traversed the vessel wall. The post-capillary venules of nude mice are traversed by both small lymphocytes and polymorphonuclears while only small lymphocytes migrate across the vessel wall of post capillary venules from normal mice (Figs 3 and 4) The migrating leucocytes in both groups of mice are localized between the endothelial cells in long winding intercellular spaces. These spaces can practically always be traced from the leucocytes to the lumen or to the basement membrane of the vessel.

A finding observed both in normal and nude mice—but not noticed in previous studies (2, 5)—was the occurrence of a very intimate contact between some high endothelial cells and a few of the migrating lymphocytes. The phenomenon is illustrated in Figs. 6 and 7 The figures show two comparable high endothelial cells each of them with a migrating lymphocyte nearly surrounded by the cytoplasm of the endothelial cell. The lymphocyte seen in Fig 6 (conf also Figs 3 and 4) is surrounded by a narrow but definite intercellular space. This is the typical finding In contrast, the intercellular space which surrounds the lymphocyte in Fig 7 is wiped out in the area between the endothelial cell nucleus and the lymphocyte nucleus. A high magnification of this area is shown in Fig. 8. The figure clearly indicates a fusion of the glycocalyces of the two cell membranes. Fig 11 illustrates another area with an intimate contact between a migrating lymphocyte and a high endothelial cell. In this area the endothelial cell shows signs of an intense secre-



Fig 3 Post-capillary venule from a normal (nu/?) mouse. Lymphocytes (ly) traversing the vessel wall between high endothelial cells (he). 1: lumen, bm: basement membrane. × 10,000

Fig 4 Post-capillary venule from a nu/nu mouse. Lymphocytes (ly) as well as polymorphonuclears (pn) traverse the vessel wall between the normally appearing high endothelial cells (he). 1: lumen, bm: basement membrane. × 10,000

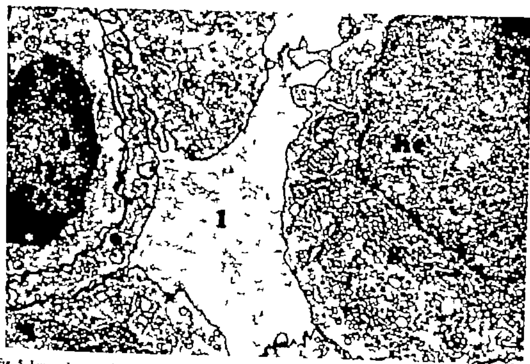


Fig 5 Luminal parts of high endothelial cells (he) from a nu/nu mouse. Note prominent Golgi apparatus (g) l lumen, ly lymphocyte.
× 20,000

tion. The secretory material is localized in 2000 Å large membrane-bound granula of varying density The secretory product is obviously liberated into the narrow intercellular space forming a material which has the same or a somewhat higher density than the glycocalyces of the cell membranes. Neither in these areas nor elsewhere in the vessel wall, the lymphocytes showed signs of pinocytosis or secretion.

A typical finding frequently observed in the HE-venules of nude mice but never noticed in normal mice, was the occurrence of degenerating leucocytes in the vessel wall. The appearance of these degenerating cells was mostly lymphocyte-like (Fig 9) but in many cases the extent of degeneration did not permit any classification of the cell (Fig 10)

DISCUSSION

The paracortical lymphocyte depletion of lymph nodes from nude mice has been described by *de Sousa et al.* (1969) In contrast to the findings by *de Sousa et al.* we also found an extreme depletion of small lymphocytes in most parts of the outer cortical areas of the lymph nodes of nude mice This discrepancy probably depends on the type of lymph node studied. Thus, the mesenteric lymph node and the deep cervical lymph nodes exhibit very well-developed outer cortical areas with numerous primary follicles and germinal centres (1)

Even in the paracortical areas of the inguinal and axillary lymph nodes which were nearly totally depleted of lymphocytes, we found many fully developed post-capillary venules with lymphocytes and polymorphonuclears traversing the vessel wall. But also thin and empty post-capillary venules—which according to the authors mentioned above are typical for the lymphocyte depleted paracortex—were seen. Our findings contrast those obtained in a recent study of post-capillary venules from lymph nodes of neonatally thymectomized mice undergoing wasting where the venules were found only occasion-


HE
HE
L

Fig 11 Two high endothelial cells (HE) one of these in close contact with a traversing lymphocyte (LY) Membrane bounded granula with diameters of about 2000 Å are seen (single arrows) These granula are obviously secreted to the inter cellular space (double arrows) Note numerous microvesicles in the cytoplasm of the endothelial cell and the total absence of pinocytotic activity in the lymphocyte. × 60,000

endothelial cells including the occurrence of secretory granula were observed in some areas of close contact between lymphocytes and endothelial cell membranes. The secretory product was obviously liberated into the inter cellular space surrounding the migrating lymphocytes. This might indicate that products from the endothelial cells are transferred to the surfaces of traversing lymphocytes as well as endothelial cells. As only a very sparse pinocytotic activity is seen in the cytoplasma of the migrating lymphocytes the material probably does not enter into the cytoplasm of the lymphocytes. In the light of a recent study (13) where immunoglobulins were demonstrated in the luminal parts of the high endothelial cells, it is tempting to speculate that the secretory material might represent receptorimmunoglobulins which are secreted to the surfaces of the endothelial cells and/or lymphocytes, thereby facilitating mutual recognition as well as the passage of lymphocytes through the walls of the post capillary venules

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HE
HE
LY

This suspension was diluted in phosphate-buffered Hank's balanced salt solution containing 0.1 per cent gelatin to a concentration of 12–18 × 10^7 colony forming units per ml.

Serum

One ml volumes of pooled fresh normal serum from six adults were stored at −30 C. Immediately prior to each experiment, 1 ml freshly thawed se rum was added to 3 ml phosphate-buffered Hank's balanced salt solution containing 0.1 per cent gela tin.

Leucocyte-Bacteria Suspension

0.5 ml leucocyte suspension 0.1 ml bacteria suspension and 0.4 ml diluted serum were added to 12 × 75 mm disposable plastic tubes. This provided about 3 bacteria per granulocyte and a final concentration of 10 per cent serum. In the tests with phenylbutazone 0.2 0.8 2 4 and 6 mg of this agent (manufactured by Geigy Basel Switzerland and used at a concentration of 200 mg per ml solution) were added to the granulocyte suspensions and diluted serum immediately before mixing with the bacteria suspensions In the control tests, only phenylbutazone was not included. The tests were incubated at 37 C with an end over end rotation to promote contact between bacteria and leucocytes. Samples were removed at prescribed intervals for the determinations of the total number of viable bacteria and the number of viabl intracellular bacteria. The bactericidal capacity of the granulocytes is proportional to the total number of bacteria killed and inversely proportional to the total number of viable bacteria or the number of viable intracellular bacteria (6) The number of bacteria phagocytized equals the number of viable intracellular bacteria plus the number of bacteria killed (6)

The total number of viable bacteria was deter mined after osmotic disruption of the leucocytes by adding 0.01 ml of the leucocyte-bacteria suspension to 1 ml distilled water Quantitation of viable bacteria was made from ppropriate dilutions of this suspension using a standard pourplate technique and Penassay aga (Difco)

The number of viable intracellular bacteria was determined by the technique of *Solberg* (6) 0.01 ml of the leucocyte-bacteria suspension and 1 ml Hank balanced salt solution containing 0.1 per cent gelatin, 500 µg streptomycin, 500 units penicillin G and 2 mg phenylbutazone were incubated at 37 C for 15 minutes and centrifuged for 10 minutes at 500 *g* The cellular pellet was twice washed in Hank's balanced salt solution and resuspended in 1 ml distilled water for osmotic disruption of the leucocytes to occur Quantitation of viable bacteria was made by the standard pour plate technique.

Controls

Controls consisted of tubes with bacteria and 10 per cent serum without leucocytes to demonstrate any direct bactericidal activity of the serum, and tubes with leucocytes and bacteria incubated without rotation to detect any extracellular bactericidal activity caused for example by enzymes liberated from damaged granulocytes. No reduc tion in viable bacteria was observed in these control tests during 1 hour's incubation.

RESULTS

In the control test, a marked reduction in viable bacteria was observed especially during the early phase of incubation (Fig. 1) de monstrating rapid phagocytosis (Fig. 2) and intracellular killing of the bacteria. By con trast, in the test containing 2 mg phenylbuta zone the majority of bacteria remained viable after incubation for 60 minutes and large numbers of the bacteria were located intra cellularly (Fig 1) indicating markedly re duced bactericidal activity of the granulocytes. Furthermore, the phagocytic activity of the granulocytes was also significantly re duced (Fig 2) After incubation for 15 minutes more than 70 per cent of the bacteria were located extracellularly in contrast to only 10 per cent in the control test. Increasing the concentration of phenylbutazone from 2 to 4 and even 6 mg per ml leucocyte-bacte ria suspension did not further influence the phagocytic and bactericidal activities of the granulocytes.

When the concentration was reduced to 0.2 mg phenylbutazone per ml leucocyte—bacteria suspension, minor reductions in nor mal phagocytic (Fig 2) and bactericidal (Fig 1) activities were observed. 0.8 mg phenylbutazone caused intermediate reduc tions in normal granulocyte functions i.e. less than 2 and more than 0.2 mg of the drug per ml leucocyte-bacteria suspension.

To study whether phenylbutazone was bound to the cells, leucocyte suspensions con taining 2 mg phenylbutazone per ml and controls containing no drug were incubated for 15 minutes at 37 C. Following incubation, the cells were twice washed in Hank's ba

2 mg PHENYLBUTAZONE
0.2 mg PHENYLBUTAZONE
CONTROL
TOTAL BACTERIA
INTRACELLULAR BACTERIA
NUMBER OF VIABLE BACTERIA
10^7
10^6
10^5
15 30 45 60
MINUTES
INCUBATION TIME

Fig. 1. Viable bacterial counts during incubation of leucocyte-bacteria suspensions with and without phenylbutazone (mean of five experiments)

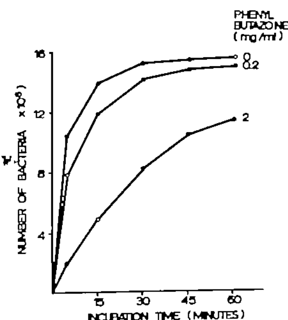


Fig. 2. Number of phagocytized bacteria during incubation of leucocyte-bacteria suspensions with and without phenylbutazone (mean of five experiments)

lanced salt solution and exposed to the bacteria in the presence of diluted serum as previously described. As visualized from Fig. 3, no permanent effect of phenylbutazone upon resting granulocytes was observed.

DISCUSSION

Phagocytosis and intracellular killing of bacteria are important physiological functions of polymorphonuclear leucocytes and several drugs have been shown to influence either of these functions (for review see 4). Evaluation of this influence requires simultaneous analyses of both phagocytic and bactericidal activities of the granulocytes. However many of the studies in the past have been confined to the phagocytosis and minor attention has been paid to the fate of intracellular bacteria (for review see 1, 2). A major problem has remained the elimination of non-phagocytized bacteria in an *in vitro* phagocytic system in order to study the dynamics of the intracellular phase (6, 7, 11). By our method, extracellular bacteria are effectively

Together with the granulocyte enzyme myeloperoxidase and a halide hydrogen peroxide form a strong antibacterial system and in guinea pig polymorphonuclear leucocytes the impaired bactericidal activity in the presence of phenylbutazone seems to be due to decreased glucose-6-phosphate dehydrogenase and hexose monophosphate shunt activity resulting in poor hydrogen peroxide formation (10) In the present study it was shown that phenylbutazone exerted no permanent effect on non phagocytizing cells suggesting that the drug either diffuses easily across the cell membrane but is not fixed to its site of action when the cells are not engaged in phagocytosis or that the membrane of non phagocytizing cells is not permable to the drug In the latter case, it would seem likely that phenylbutazone is taken up from the surrounding medium along with the ingested particles. In previous studies however when extracellular bacteria were eliminated after incubation of the leucocyte-bacteria suspension for only few minutes and the granulocytes still possessed marked bactericidal activity phenylbutazone immediately stopped the killing of already phagocytized bacteria (8) indicating that the drug is not only taken up along with the ingested particles but may easily diffuse across the cell membrane. This is also supported by the results of our test for the determination of the number of viable intracellular bacteria. In this test, the concentrations of granulocytes and bacteria are so small (for the granulocytes 1/100 of the concentration in the original leucocyte-bacteria suspension and for the bacteria even less) that virtually no phagocytosis can take place (6) However phenylbutazone in the surrounding medium immediately inhibits the killing of intracellular bacteria (6, 8)

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SELECTIVE DEFECTS OF *IN VITRO* PARAMETERS OF LYMPHOID CELL FUNCTION IN PATIENTS WITH IRRADIATED TESTICULAR TUMOUR AND IN PATIENTS WITH LYMPHOPROLIFERATIVE DISORDERS

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The capacity of lymphoid cells to manifest *in vitro* cytotoxicity against chromium-labelled chicken erythrocytes in the presence of PHA or antibodies to chicken erythrocytes was studied in 24 patients with testicular tumours who had received extended abdominal field irradiation, and in five patients with malignant lymphomas. The PHA-induced cytotoxicity in patients with irradiated testicular tumour was significantly lower than that in the normal controls, while no significant difference in antibody-induced cytotoxicity was found. In four of the patients, a markedly impaired cytotoxic response to PHA was found in spite of normal DNA synthesis induced by this mitogen. Two patients failed to respond to PHA both by cytotoxicity and DNA synthesis. A deficient PHA-induced cytotoxicity but normal cytotoxic activity in the presence of anti-target cell antibodies, was observed in six of the patients. These dissociations in terms of *in vitro* parameters of lymphocyte function may reflect selective depression of lymphocyte subpopulations resulting from radiotherapy or disease processes.

During the last decade, a number of mechanisms have been described whereby lymphoid cells may destroy target cells *in vitro* (19). Following the recognition of ontogenetically and functionally distinct subpopulations of lymphocytes, considerable interest has centered around the delineation of the different lymphocyte classes mediating these reactions. Thus, in the mouse, where experimental procedures allowing the study of relatively pure lymphocyte subpopulations have been developed, evidence has been provided that thymus-dependent lymphocytes (T-cells) are responsible for cytotoxicity against allogeneic cells (3), while thymus-independent lymphocytes seem to mediate the cytotoxicity induced by anti-target cell antibodies (9) and the cytotoxic effect on certain tumour cells (13).

For obvious reasons, corresponding experiments cannot be performed on man. However, important data regarding lymphocyte function in immunological reactions may be derived from the study of patients with inborn or acquired disease states affecting the

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immune system. We have studied the *in vitro* cytotoxic activity of lymphoid cells from patients with lymphoproliferative disorders and patients who have received radiotherapy in the course of treatment of testicular tumour. The results of this investigation may contribute to the elucidation of the role of human lymphocyte subpopulations in immunological reactions *in vitro*.

MATERIALS AND METHODS

Lymphocyte Donors

24 patients with testicular tumours, and 5 patients with malignant lymphomas were studied during the period January–March 1973. The patients with testicular tumour had received extended abdominal field irradiation. In 9 cases the mediastinum was included in the irradiation field. The patients studied form part of a larger series of patients whose immunological functions are being investigated. Normal lymphocytes were obtained from blood donors.

Separation of Lymphocytes from Peripheral Blood

Lymphocytes were separated from heparinized venous blood by the Isopaque-Ficoll gradient centrifugation technique (2). The cells were washed three times in Hanks balanced salt solution (HBSS, Grand Island Biological Company, New York), counted in a Burker chamber and suspended in Medium 199 (Grand Island Biological Company) containing penicillin (50 IU/ml), streptomycin (50 μg/ml) and 3 per cent heat-inactivated foetal bovine serum (FBS, Flow Laboratories, Ayrshire, Scotland) at a concentration of 5×10^6 per ml. The cells obtained in this manner consisted, on the average, of 90 per cent lymphocytes, 8 per cent monocytes and 2 per cent granulocytes, as assessed by light microscopy of cytocentrifuge-made preparations stained with May-Grünwald-Giemsa. As judged by the ability to exclude trypan blue, 98–100 per cent of the cells were viable.

Cytotoxicity Assay

Cell-mediated cytotoxicity was induced by phytohaemagglutinin (12) or anti-target cell antibodies (16) and measured by an isotope release technique using chicken erythrocytes as target cells (20). Blood was drawn into a heparinized syringe from wing veins of White Leghorn chickens, 4–12 weeks old. About 1×10^8 erythrocytes in 0.1 ml of Medium 199 were mixed with 0.1 ml isotonic sodium chromate solution, specific activity 100–300 mCi/mg Cr (Isotope Laboratories, Kjeller, Norway) and incubated at 37° C for one h, washed twice in HBSS and finally suspended in culture medium with FBS and antibiotics at a concentration of 2×10^5 per ml.

For the cytotoxicity assay, 2.5×10^6 effector cells in 0.5 ml medium with FBS and antibiotics were mixed with 1×10^5 ^{51}Cr-labelled target cells. 0.5 ml of a 1:25 dilution of phytohaemagglutinin (PHA MR 10, Wellcome Research Laboratories, Beckenham, England) (29) or 0.5 ml of a 3×10^{-4} dilution of heat-inactivated rabbit anti-chicken erythrocyte antiserum (28) was then added. In control cultures, medium alone or diluted normal rabbit serum was used instead of PHA and antiserum, respectively. A large excess (1×10^7) of unlabelled chicken erythrocytes served as control cells for the effector lymphocytes.

Patients and normal blood donors were always tested in the same experiments.

The tubes were incubated for 22 h at 37° C in an atmosphere of 5 per cent CO_2 and 100 per cent humidity. They were then centrifuged for 10 min at 150 *g* and the radioactivity of the supernatant and the total radioactivity of each tube were measured in a gamma ray counter (NE 8312, Nuclear Enterprises, Edinburgh). After subtraction of the background, the results were presented as the Cytotoxic Index, which is an expression of the amount of radioactivity released from the target cells during incubation.

$$\text{Cytotoxic Index (CI)} = \frac{\text{Radioactivity of the supernatant}}{\text{Total radioactivity}} \times 100$$

Total lysis of the chicken erythrocytes (with distilled water) corresponds to a Cytotoxic Index of about 90. When the CI of the control tubes with medium or normal rabbit serum are subtracted from the CI of those with PHA and antiserum, respectively, the values obtained are termed Corrected Cytotoxic Index. The results given are the means of duplicates. The difference between duplicate CI values rarely exceeded 3.0.

Lymphocyte Stimulation in Culture

Lymphocyte stimulation by PHA was measured by the incorporation of ^{3}H labelled thymidine into DNA, essentially as described, in triplicate cultures (5). A total cell number of 1×10^6 was used in a volume of 1.1 ml, with PHA present in a final concentration corresponding to the concentration in the cytotoxicity cultures. Control cultures without PHA were set up in parallel. Harvesting and liquid scintillation counting were performed as described (5). The results are presented as disintegrations per minute (DPM).

TABLE 1 *Cell-mediated Cytotoxicity of Patients and Normal Controls Induced by PHA and Anti-target Cell Antibodies*

Subjects tested	Cytotoxic Index (mean and range) with: PHA	Medium	Antiserum	Norm. rabbit serum
Normal blood donors (n = 30)	38.7 (22.2–55.7)	6.0 (3.0–10.2)	39.2 (23.6–62.7)	6.1 (2.7–9.9)
Lymphoma patients (n = 5)	18.2 (9.8–48.4)	6.6 (2.8–9.9)	31.2 (18.6–42.9)	6.4 (2.3–11.5)
Patients with testicular tumour (n = 24)	32.7* (10.5–53.7)	6.6 (2.8–13.1)	36.8 (18.7–58.0)	6.4 (3.2–12.0)
Control cells† (n = 15)	4.7 (1.8–8.5)	4.7 (2.0–8.6)	4.9 (1.9–9.8)	4.8 (1.8–8.4)

* n = 21
† control cells = 1×10^{5} chicken erythrocytes.

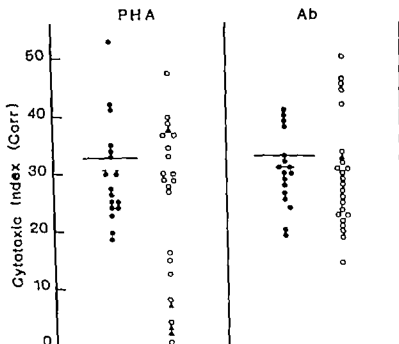


Fig 1 Cell-mediated cytotoxicity induced by PHA or anti-target cell antibodies (Ab) in patients with irradiated testicular tumour (O) patients with lymphoma (▲) and in normal controls (●) ——— = mean, – – – – – = median.

RESULTS

A summary of the cytotoxicity experiments is given in Table 1 and in Fig 1 the results obtained with lymphocytes from all the patients and normal controls are shown in a scatter diagram. Statistical analysis of the data showed that the PHA-induced cytotoxicity in patients with irradiated testicular tumour was significantly lower than that in the normal controls ($p<0.05$ Wilcoxon rank

TABLE 2. *Defects of Cell-mediated Cytotoxicity in Patients with Irradiated Testicular Tumour and Patients with Malignant Lymphomas. Pathological Values in Italics*

Patient	Diagnosis	Treatment	Cytotoxic Index* with PHA	Cytotoxic Index* with Antiserum	DNA synthesis (DPM)§
E. N	Test. tumour	Irrad. 1968	*12.5*	24.8	148 475
M. L.	Test. tumour	Irrad. 1968	*4 1*	22.6	109 766
W S	Test. tumour	Irrad. 1962	*0 1*	21 0	227 616
O H	Test. tumour	Irrad. 1972	*8.0*	19.8	n.t.†
N S	Lymphosarcoma (generalized)	None	*5.5*	35.1	571 047
O P	Hodgkin's dis. (stage II B)	None	*3.5*	*12.2*	*8 926*
V S	CLL‡	None	*2.9*	*11.5*	*11 828*
H A.	CLL	None	*7.5*	32.5	n.t.
Normal donors (n = 30)			32.7 (18.2–47 1)	35.1 (19.5–55 7)	100 000 – 600 000

* Corrected Cytotoxic Index.
§ Values of unstimulated cultures have been subtracted.
† n.t. = not tested.
‡ CLL = chronic lymphatic leukaemia.

sum test) while no significant difference in antibody-induced cytotoxicity was found. The data on the heterogeneous lymphoma group were not subjected to statistical analysis.

In Table 2 the diagnosis and treatment of the individual patients with abnormal *in vitro* cytotoxicity are given together with the immunological parameters determined. The results reveal several different patterns of defects of *in vitro* parameters of lymphocyte functions

a) *Dissociation between Cytotoxicity and DNA Synthesis Induced by PHA*

In four patients (E.N M.L., W.S and N.S.) a markedly impaired cytotoxic response to PHA was found in spite of normal DNA synthesis induced by this mitogen.

b) *Deficiency of both Cytotoxicity and DNA Synthesis Induced by PHA*

Two patients (O.P and V.S.) failed to respond to PHA both by cytotoxicity and DNA synthesis. These two, both lymphoma patients also had an abnormally low antibody induced cytotoxicity

c) *Dissociation between Cytotoxicity Induced by PHA and Anti-target Cell Antibodies*

An impaired PHA induced cytotoxicity but normal cytotoxic activity in the presence of anti-target cell antibodies, was observed in six patients (E.N M.L., W.S O.H. N.S. and H.A.)

DISCUSSION

In the present investigation, dissociations in terms of *in vitro* parameters of lymphoid cell functions have been demonstrated in patients with malignant lymphoma as well as in patients with testicular tumour who had received extended abdominal field irradiation.

In some of the patients, a markedly impaired PHA induced cytotoxicity was found in spite of normal DNA synthesis induced by this mitogen in optimal concentrations. A similar phenomenon in three patients with "acquired" agammaglobulinaemia who had a low PHA induced cytotoxicity and a quantitatively normal although delayed DNA synthesis in response to this mitogen has been reported (14) A dissociation in the opposite

direction" i.e. relatively strong cytotoxic response to PHA, but low blastoid transformation in certain patients with malignant lymphomas has been reported (11) We have observed a similar pattern in a young girl (B.R.) with chronic mucocutaneous candidiasis, whose lymphoid cells manifest normal *in vitro* cytotoxicity in response to concanavalin A, although they fail to synthesize DNA (data not published) Recently it was reported that leucocyte cultures from certain patients with Wiskott Aldrich syndrome produced lymphotoxin in the absence of significant lymphocyte transformation (17) Thus, a dissociation between mitogen-induced cytotoxicity and DNA synthesis apparently occurs in a number of disorders affecting the immune system This phenomenon may be related to the observation that, in normal lymphocytes, nucleic acid synthesis can be blocked without affecting PHA induced cytotoxicity (10 15)

The dissociation between cytotoxicity and DNA synthesis might be explained by two different theories. Either these two effects of mitogen stimulation may be independent expressions of activation of the same lymphocytes, or they may represent separate responses of two different subpopulations of lymphocytes each stimulated by PHA. The latter hypothesis is in line with recent data suggesting that, upon PHA stimulation, lymphotoxin is secreted by a subpopulation of T lymphocytes which is separate from the cells active in DNA synthesis (22) Dissociations in terms of lymphocyte subpopulation activity have also been described for DNA synthesis and the secretion of other "lymphokines" (21 26) Support for this theory is also derived from the observation that thymus cells may be transformed by concanavalin A without acquiring cytolytic capacity (23)

Another characteristic defect found in some of our patients was a dissociation between cytotoxicity induced by PHA and anti-target cell antibodies In accordance with the reasoning above, we feel that this phenomenon is most easily explained by assuming that these two cytotoxic reactions are mediated by separate populations of lymphocytes. There is, in fact, convincing evidence that the cells responsible for antibody-dependent cytotoxicity are thymus-independent, although recent data suggest that they may not be B-lymphocytes (8, 28) It is tempting to speculate that they may belong to a population of lymphocytic cells lacking surface markers of both T and B-lymphocytes, that has been shown to exist in both mice (25) and man (7) Less is known about the identity of the cells mediating PHA induced cytotoxicity although thymus-dependent lymphocytes seem to be involved (1)

The dissociations described may reflect selective depression of lymphocyte subpopulations as a result of disease processes or treatment particularly radiotherapy Indeed, radiotherapy has been shown to lead to a lymphopenia mainly affecting T lymphocytes (24) The effect of radiotherapy on lymphocyte subpopulations may be of long duration, as some of the patients included in the present study and earlier investigations (4) had received their radiotherapy several years ago The effect of radiotherapy on cytotoxic lymphocytes is demonstrated by our observation that patients with irradiated testicular tumour had significantly lower PHA induced cytotoxicity than the normal controls, although no significant difference in antibody induced cytotoxicity could be found. It is of interest to note that the *in vitro* cytotoxicity of the 9 patients whose thymus had been included in the irradiation field did not differ significantly from the others. A significant depression of cytotoxicity after irradiation for bladder cancer (18) as well as for breast cancer (27) has also been described.

Regarding the lymphocyte deficiencies in patients with untreated lymphoma, several possibilities may be considered. The malignant process may primarily affect the subpopulation responsible for the *in vitro* lymphocyte function studied, compromising its activity Alternatively the function of a lymphocyte subpopulation may be disturbed by the malignant growth of other lymphoid cells.

Lymphocytes from the patients and normal controls included in the present investigation

were also tested for the presence of membrane-bound immunoglobulin a marker for human B-lymphocytes (6) A slightly negative correlation between the percentage of Ig positive lymphocytes and PHA induced cytotoxicity was found, but there was no correlation between the number of Ig positive lymphocytes and antibody-induced cytotoxicity (data not given) While these findings do lend some support to the theories of the nature of the effector cells outlined above, it is evident that data of this kind must be interpreted with caution

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MATERIALS AND METHODS

Preparation of ³H-mitomycin C

Mitomycin C (Kyowa Hakko Kogyo Co. Ltd., Tokyo) tritiated by the Wilzbach method, was obtained from the Radiochemical Centre Amersham as lyophilised material from which labile tritium and catalyst had been removed. Since the tritiation procedure caused some degradation of the antibiotic, the crude radioactive product was purified by the following procedure. A small portion of the freeze-dried material was dissolved in methanol and chromatographed on paper for 20 hours using a benzene/methanol/phosphate buffer solvent (*Lefemine et al.* 196) The mitomycin band remaining near the site of application was eluted with distilled water and rechromatographed on paper using distilled water adjusted by NH_3 to pH 6.9 as solvent. Mitomycin C with an Rf value of approximately 0.7 in this system, was eluted from the paper with distilled water and the solution was cleared by filtration (Millipore 10 mµ)

The final product showed the same absorption spectrum as non-radioactive mitomycin C and, in different samples, the specific activity varied from 60 to 140 10^3 cpm/µg of purified antibiotic. Purification of ³H-mitomycin C was also carried out according to *Weissbach & Lisio* (1965) The specific activities obtained from a given sample were similar using both methods, but the procedure described above gave substantially higher yields. Purified ³H-mitomycin C was stored for maximum 3 hours before use in cell culture experiments.

Incubation with Radioactively Labelled Compounds

Mouse P 388 cells grown in suspension culture (*Østerud* 1972a) were used throughout this study and in experiments designed to study release of H-mitomycin C, cells prelabelled with ¹⁴C-thymidine were employed.

Labelling with ¹⁴C-thymidine was performed during 24 hours preceeding exposure to ³H-mitomycin C. The cultures were started at an initial density of 0.5 10^5 cells/ml and a total of approximately 10^{-11} Ci/ml of ¹⁴C-thymidine was added in 4 portions at 6-hour intervals. The ¹⁴C-labelled cells were harvested by centrifugation at 37 C and resuspended at a density of 0.5–0.8 10^6 cells/ml in fresh, non-radioactive growth medium.

The cultures were then immediately supplemented with ³H-mitomycin C to final concentrations varying from 0.5 to 50 µg/ml, and incubated on shake flasks at 37 C for 1 hour

In experiments including post-treatment incubation, the mitomycin C treated cells were harvested by centrifugation, washed twice in medium (37 C) suspended in fresh, non-radioactive growth medium and incubated on shake flasks. This procedure reduced the amount of H-radioactivity in the culture medium to less than 0.01 per cent.

Fractionation of Cell Cultures

Culture samples were removed at intervals after exposure to mitomycin C. The cells were harvested by centrifugation at +4 C, and washed 4 times with cold SSC* Washed cells were then either dissolved in 1 N KOH, or fractionated by the following procedure

The cold acid soluble cell fraction was obtained by extraction at 0° C for 20 minutes with 5 per cent TCA (w/v) followed by washing twice with cold TCA.

The lipid fraction was extracted at 46 C for 30 minutes with 75 per cent ethanol/ethyl ether (1 1) and the cell residue was washed with cold SSC.

The RNA fraction was obtained by alkaline hydrolysis. After incubation in 0.3 N KOH at 37 C for 15 hours, the hydrolysate was cooled at 0° C, acidified by 0.2 volumes of 6 N HCl and 1 volume of 5 per cent TCA, and centrifuged at +4 C and 3000 × *g* for 15 minutes. The cell pellet was washed with cold TCA.

The DNA fraction was obtained by extracting twice at 90 C for 20 minutes with 5 per cent TCA.

The acid insoluble protein fraction was finally dissolved in 1 N NaOH.

Colorimetric Determination of Nucleic Acids

RNA was measured as orcinol reacting material against ribose (*Brown* 1946) DNA as diphenylamine reacting material against deoxyribose (*Burton* 1956) More than 98 per cent of the RNA was found in the acidified KOH hydrolysate and the subsequent cold TCA washing, more than 99 per cent of the DNA was found in the hot TCA cell extracts.

Radioactivity Measurements

Samples for counting were prepared by adding 0.1 to 1 ml of the appropriate cell fractions or of culture medium to 15 ml of Insta-Gel® scintillation liquid (Packard Instrument Co. Inc.) When alkaline samples were counted, the scintillation

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* Abbreviations: MC Mitomycin C ¹⁴C-TdR Thymidine 2-¹⁴C, specific activity 62 mCi/m mol, obtained as sterilized aqueous solution from the Radiochemical Centre, Amersham, and stored at +4 C for maximum 2 months. SSC: sodium chloride (0.15 M) and tri-sodium citrate (0.02 M) pH 7.2, TCA: trichloroacetic acid, cpm. counts per minute, dpm: disintegrations per minute.

fluid was supplemented with 3 ml/l of concentrated acetic acid. Radioactivity was measured in a Packard Tri-Carb model 3365 liquid scintillation counter with counting efficiencies of 20–25 per cent for ³H and 60–65 per cent for ¹⁴C. Calculation of dpm in doubly labelled samples was carried out according to *Peas* (1973)

RESULTS

Cellular Uptake and Intracellular Distribution of ³H mitomycin C

Growing cultures of P 388 cells were supplemented with ³H mitomycin C and incubated in the shaker at 37° C for 1 hour The cells were then harvested and the content of ³H radioactivity was measured in the culture medium and in alkaline lysates of whole washed cells. As shown in Fig 1 the cellular uptake of radioactivity was proportional to the extracellular concentration of the antibiotic over the concentration range 0.5–50 µg/ml of ³H mitomycin C It was consistently found that only about 0.01 per cent of the added ³H mitomycin C was incorporated into the cells.

TABLE 1 *Localization of Intracellular ³H-mitomycin C*

Cell fraction	Radioactivity (per cent of total cell bound) mean	range
Cold TCA soluble	59	53–65
Lipid fraction	0.5	0–4
RNA fraction	22	13–25
DNA fraction	9	7–13
Protein fraction	9	7–10

* Four separate cultures with concentrations of ³H mitomycin C varying from 0.5 to 50 µg/ml.

Mouse P 388 cells were grown in medium containing ³H-mitomycin C for 1 hour harvested and washed. The amounts of radioactivity in various cell extracts were measured by liquid scintillation counting

The intracellular localization of ³H mitomycin C was studied by measuring the amounts of ³H radioactivity in the cell fractions containing cold TCA soluble material, lipids, RNA, DNA and acid insoluble protein From the data presented in Table 1 it can be seen that approximately 60 per cent of the intracellular radioactive material was found in the cold TCA soluble cell fraction It further appeared that, among the cell fractions *containing macromolecules*, the RNA fraction exhibited the highest radioactivity Significant amounts of tritium appeared also in the DNA and protein containing cell fractions, whereas only small amounts were found in the lipid fraction

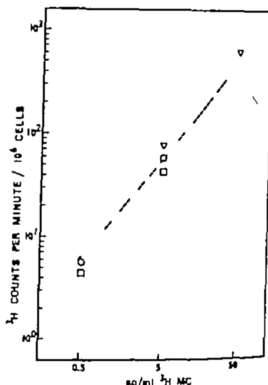


Fig 1 Uptake of ³H-mitomycin C by mouse P-388 cells. Suspension cultures containing ³H-mitomycin C in the concentrations indicated were incubated at 37° C for 1 hour. The cells were harvested, washed 4 times in cold SSC and lysed in alkali Radioactivity in cell lysates was measured by liquid scintillation counting. The graph includes results from 3 separate experiments as indicated by different symbols.

Release of ³H mitomycin C During Post-treatment Incubation

To establish whether ³H mitomycin C was lost from the P-388 cells after exposure to

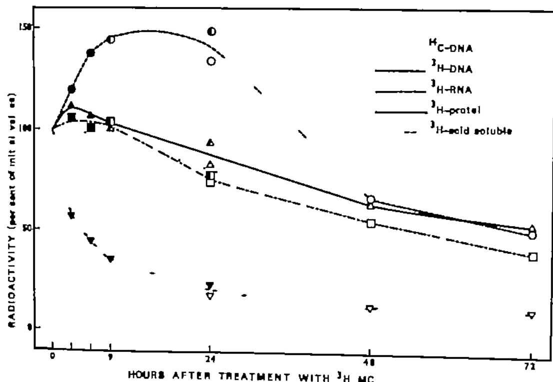


Fig. 2. Loss of radioactivity from various P-388 cell fractions with time after exposure to ^{3}H-mitomycin C. Cells prelabelled with ^{14}C-TdR were exposed to ^{3}H-mitomycin C for 1 hour and grown further in non-radioactive medium. At intervals after treatment with ^{3}H MC, ^{3}H and ^{14}C-radioactivities in the cold TCA soluble, RNA-containing, DNA-containing and protein-containing cell extracts were assayed. The graph is based on data obtained in 3 separate experiments as indicated by closed, partly closed and open symbols. The values obtained at zero time (15 minutes after transfer to non-radioactive medium) in each experiment have been normalized to 100.

the antibiotic, double labelling experiments were carried out. The cells were first grown for 1 day in medium containing ^{14}C-thymidine to label the cell DNA, then exposed to 5 µg/ml of ^{3}H-mitomycin C for 1 hour. The doubly labelled cells were subsequently washed and transferred to non radioactive growth medium and incubated at 37 C. ^{3}H and ^{14}C radioactivities in the culture medium and in the various cell fractions were assayed at intervals after exposure to the antibiotic.

Fig. 2 illustrates the patterns of ^{3}H-release from the cold acid soluble cell fraction and from the cell fractions containing DNA, RNA and acid insoluble protein. Release of ^{14}C from the DNA fraction in the same cultures has been included as a reference. The graph is based on values obtained in 3 separate experiments, and the content of radioactivity in each fraction immediately after removal of ^{3}H mitomycin C from the medium has arbitrarily been set to 100.

The figure demonstrates a rapid initial loss of ^{3}H radioactivity from the cold acid soluble fraction, followed by a moderate decrease upon prolonged incubation. The amount of ^{3}H radioactivity found in the DNA containing cell fraction increased for several hours after the cells had been transferred to non-radioactive growth medium and one day after treatment with the antibiotic, the increment corresponded to 30–50 per cent of the initial value. However during the second day of post treatment incubation, a substantial drop in ^{3}H-radioactivity occured in the DNA fraction. The drop was far more extensive

TABLE 2. *Distribution of ^3H-radioactivity in P-388 Cell Cultures at Intervals after Treatment with ^3H mitomycin for 1 hour*

Cell culture fraction	Radioactivity (dpm ^3H 10^{-3}) Days after treatment			
	0	1	2	3
Culture medium	28.8	48.8	65.2	69.6
Cold TCA soluble	21.2	4.0	3.2	2.4
RNA fraction	20.8	15.2	11.2	8.0
DNA fraction	11.2	15.2	7.6	5.6
Protein fraction	12.8	10 4	8.0	6.8

Mouse P 388 cells were grown in medium containing 5 μg/ml of ^3H-mitomycin C for 1 hour washed twice in medium at 37 C, and incubated in non-radioactive growth medium at a density of 0.5 10^6 cells/ml. Two parallel samples (170 ml) were collected after equilibration in the shaker for 15 minutes, and after 1 2 and 3 days.

than the corresponding decrease in ^{14}C-radioactivity in the same extracts. Further loss of ^3H-radioactivity from the DNA fraction during the third day paralleled the loss of ^{14}C. The ^3H radioactivity exhibited by cell fractions containing RNA and acid insoluble protein also seemed to increase slightly shortly after the cells had been transferred to non radioactive medium but the significance of these differences is doubtful Judged by the values obtained after 1 2 and 3 days, the loss of ^3H radioactivity from these cell fractions roughly paralleled the loss of ^{14}C-radioactivity from DNA.

Table 2 shows the absolute dpm ^3H values obtained in one experiment, also including the ^3H radioactivity found in the culture medium. It appears from these figures that loss ^3H-radioactivity from the various cell fractions can be accounted for by a corresponding increase in the medium.

DISCUSSION

The present study demonstrated a dose dependent uptake of ^3H mitomycin C in growing mouse P 388 cells (Fig 1) Concentrations of mitomycin C varying from 0.5 to 50 μg/ml culture were employed and, based on the present data, the average amount of antibiotic incorporated seemed to be in the range of 10^4 to 10^6 molecules/cell depending on the extracellular concentration. In view of previous data on P-388 cell volume (*Ørstavik* 1972a) it appears that the concentration of antibiotic was substantially lower within the cells than in the surrounding medium. A low uptake of radioactively labelled mitomycin has previously been observed in growing bacteria (*Lino & Weisbach* 1965) the present results might suggest that the uptake in growing mammalian cells is even lower

Analysis of the intracellular distribution of ^3H mitomycin C (Table 1) revealed that, after treatment for 1 hour most of the radioactivity could be extracted with cold acid. The nature of this acid soluble material was not further investigated, but might reflect accumulation of unreacted antibiotic in the cell pool Continued binding of ^3H to cell DNA (and possibly also to RNA and protein) for several hours after the mitomycin C treated cells had been transferred to non-radioactive medium (Fig 2) is compatible with this view

It further appeared that nucleic acids and proteins both were targets for mitomycin C in growing P 388 cells, since the DNA-, RNA and protein containing cell fractions all exhibited significant ^3H-radioactivity (Tables 1 and 2) The observation that only DNA bound considerable amounts of ^3H also after the cells had been transferred to non-radioactive growth medium (Fig 2) cannot be explained on the basis of the present data. It might, however be due to the existence of

different intracellular pools of ^{3}H mitomycin C. Binding to RNA and protein might depend on a cytoplasmic pool which was likely to be released into the surrounding medium (Table 2, Fig 2) while DNA drew upon a more stable nuclear pool of mitomycin C.

The present data does not allow exact quantitative estimation of the amounts of mitomycin C bound per unit of cell macromolecules, but determination of total DNA and ^{3}H radioactivity within the same cell fraction suggested binding of 1 molecule of mitomycin C per 10^3 nucleotide pairs as an approximate value after exposure to 5 µg/ml of the antibiotic for 1 hour

Judged by the data in Table 1 as compared with the relative content of DNA, RNA and protein in P 388 cells (*Ørstavik* 1972b) it seemed that DNA and RNA bound approximately the same amount of antibiotic per weight unit, whereas the ratio ^{3}H/weight unit was 20 to 30 times less for protein. These findings correspond with previous data (*Szybalski & Iyer* 1964 *Weissbach & Lisio* 1965) on the degree of alkylation by mitomycins of different DNA, RNA and protein preparations *in vitro* Preferential reaction with nucleic acids indicated by the present data seem to suggest other preferred sites of alkylation in the P 388 cells than those indicated for *E coli* bacteria (*Lisio & Weissbach* 1965) where 60–80 per cent of cell bound mitomycins apparently were attached to proteins.

The present data also seem to suggest that a mechanism existed in the P 388 cells for the excision of mitomycin C induced DNA alkylations (Table 2 Fig 2) The results obtained during the first hours after the cells had been treated with mitomycin C are inconclusive continued binding of ^{3}H to DNA in this period might mask a concomitant minor release. However an extensive loss of ^{3}H-radioactivity from the DNA fraction, with only a moderate corresponding decrease in ^{14}C reference label during the second day after treatment, shows that mitomycin C (or a derivative thereof) was selectively removed from DNA in this period. Apparently this process was terminated after 2 days since loss of ^{3}H from the DNA fraction paralleled the loss of ^{14}C after this time and, accordingly could be ascribed to cell death.

Considering our previous observation that mitomycin C treated P-388 cells perform non semiconservative DNA synthesis (*Ørstavik* 1973) it is tempting to suggest that the selective removal of mitomycin C derived ^{3}H from DNA in the present experiments was part of a DNA repair process. DNA repair provides a feasible explanation of the mechanism whereby mammalian cells may recover from growth inhibition induced by a short treatment with mitomycin C (*Rauth et al* 1970 *Ørstavik* 1972a c) This interpretation is in accordance with previous findings in bacteria (*Boyce & Howard Flanders* 1964 *Terawaki & Greenberg* 1966) where resistance to mitomycin C was related to the ability of the organism to perform DNA repair

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LEUCOCYTE MIGRATION TEST IN AGAROSE

The Use of Puromycin in Disclosure of Non Immunological Inhibition

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The response of peripheral human leucocytes to PPD and tubercle bacilli was studied in the leucocyte migration test in agarose medium. At PPD 50 μg/ml migration of leucocytes from tuberculin positive persons was inhibited in all cases tested, while no migration inhibition of cells from tuberculin negative persons was found. There was no overlapping between the MI of the two groups. Blocking of protein synthesis by means of puromycin prevented the migration inhibition of cells from tuberculin positive persons at PPD 50 μg/ml and 100 μg/ml. In some cases the migration of cells from tuberculin negative persons was inhibited in the presence of PPD 100 μg/ml. This inhibition could not be prevented by puromycin and was probably due to a toxic effect of PPD It is suggested that puromycin may be useful in dislocating false, probably toxic, inhibition.

Recently the leucocyte migration inhibition test using capillary tubes has gained acceptance as a means by which to demonstrate cellular hypersensitivity (3 8) The test has been applied in investigations of several patient categories and it seems useful in the early diagnosis of transplant rejection (10 22) *Bendixen* (2) *and Nerup et al.* (20) have demonstrated migration inhibition of cells from patients with autoimmune diseases in the presence of autoantigens. Cells from some patients with malignant growths exhibit migration inhibition when confronted with tumour antigen (1)

Various modifications of the original capillary technique have been introduced.

Federlin et al. (11) used microcapillaries and *Hughes* (12) advocated the use of micro polythene tubing instead of glass capillaries. These modifications have the advantage of being less cell consuming and to some extent even antigen saving. Further advantage in this respect seems to emerge from the agar technique introduced by *Carpenter et al.* (4) and adopted to clinical investigations on man by *Clausen* (6)

The reliability of the test has been investigated in many laboratories where the skin reactions to various antigens have been correlated with the results of the leucocyte migration test. In most cases the reaction to tuberculin has been investigated. Some investigators found no correlation between the *in vivo* and the *in vitro* results (6 13, 14) Others found correlation between the skin reaction and the migration inhibition (6, 7 11 17 19 21) although most investigators had to face the problem of overlapping between the migration indices (MI) of the

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tuberculin positive and the tuberculin negative group due to nonspecific inhibition in the negative group

According to a number of reports (9 15 18) migration inhibition in the capillary technique is dependent on the intact protein synthesis of the migrating cell population. Puromycin at a concentration that interferes with protein synthesis prevents the PPD induced migration inhibition of tuberculin sensitive cells (9 18) The present work is concerned with the use of puromycin for the solution of the problem of false inhibition of nonsensitized cells at high PPD concentrations in the agarose technique, slightly modified according to *Clausen* (6)

MATERIALS AND METHODS

Healthy adults of both sexes were tested with Mantoux II (0.2 mg PPD/ml) Skin induration exceeding 8 mm was considered positive reaction. Healthy children of both sexes giving a negative Moro reaction served as a tuberculin negative group

Cell isolation 8 ml of venous blood were collected in 10 ml plastic tubes (Cat. no. 1080 Nunc, Denmark) containing 250 units of preservative-free heparin and 2 ml of 5 per cent W/v dextran T250 (Pharmacia) dissolved in 0.88 per cent NaCl. After 45 minutes of sedimentation at 37 C, the leucocyte rich plasma was pipetted off centrifuged at 220 g and the cells were washed three times in Hanks Balanced Salt Solution by centrifugation at 220 g for 5 minutes. Finally the cells were resuspended in TC–199 (Flow Lab. Scotland) with 10 per cent preservative-free horse serum (Statens Seruminstitut, Denmark) and streptomycin penicillin 100 u./ml (Flow Lab USA) The cell concentration was adjusted to 2×10^{8}/ml.

Antigen Preservative-free purified protein derivative (PPD) (1 mg/ml) in phosphate buffer and a heat killed suspension of tubercle bacilli in phosphate buffer (1.8×10^{8} bacilli/ml) were supplied by Statens Seruminstitut, Denmark.

Agar Medium Fresh agar medium was prepared every day For the production of 100 ml agar medium 1 g agarose (L'Industrie Biologique Française, France) was added to 74 ml distilled water and heated to 100 C. After cooling to 50 C 10 ml TC–199 10 x, 10 ml horse serum, 1 ml M Hepes buffer (Flow Lab.) 3 ml 5 6 per cent W/v $NaHCO_3$, and 2 ml streptomycin-penicillin were added. pH was adjusted to 7.2–7 4 by addition of 3.5 N NaOH. The agar was poured into disposable plastic trays (Partigen Trays, Behringwerke) 10 ml in each and 12 holes (diameter 3 mm) were punched in the agar gel.

Migration technique The cell suspension was incubated for 30 minutes at 37 C in sealed polypropylene micro test tubes (400 µl, Miflan Instruments, Switzerland) half of the cell suspension in the presence of antigen, the other half without antigen serving as control. In some experiments the corresponding amount of phosphate buffer was added to the control cells. The MI of these cultures did not differ from the MI of cells incubated with TC-199 alone. During incubation, the cells were resuspended every 10 minutes to facilitate the maximal contact between cells and antigen. The agar plates were prewarmed at 37° C for 30 minutes before application of the leucocyte suspension, 7 µl in each hole. To determine the MI for one person, 6 holes with antigen and 6 holes without antigen were used. After 24 hours' incubation at 37 C in an athmosphere of 5 per cent CO_2, 95 per cent athmospheric air saturated with water vapour the migration areas were read in a projection microscope and measured by paper planimetry. The migration index (MI) was calculated according to the formula

$$\text{MI} = \frac{\text{Migration area with antigen}}{\text{Migration area without antigen}} \times 100$$

Puromycin (Calbiochem, USA) was dissolved in TC–199 with horse serum 10 per cent and antibiotics, at a concentration of 1 mg/ml, and stored at – 20 C until used. For the investigation of the effect of puromycin, four tubes were parallelly incubated for 30 minutes at 37 C one tube containing puromycin in the presence of PPD one with puromycin alone, one with PPD alone, and one tube containing cell suspension without any additives.

The puromycin migration index was calculated according to the formula.

$$\frac{\text{Migration area with antigen and puromycin}}{\text{Migration area with puromycin alone}} \times 100$$

RESULTS

Preliminary studies showed that PPD 50 µg/ml was optimal for discrimination between a tuberculin positive and a tuberculin negative group

If cells from 18 Mantoux positive and 14 Moro negative persons were applied, the mean MI at PPD 50 µg/ml would be 60 (SD 11) and 99 (SD 1) respectively (Fig 1) On the basis of these figures, the MI discrimination between inhibition and non-in-

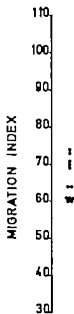


Fig 1

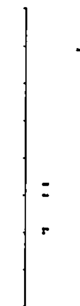


Fig 2

Fig 1 The leucocyte migration test in 18 tuberculin positive ○ and 14 tuberculin negative ● persons, using PPD 50 μg/ ml as antigen.

Fig 2. The leucocyte migration test in tuberculin positive ○ and negative ● persons, using particulate antigen (heat killed tubercle bacilli at contrations of 9×10 bacilli/ml)

hibition at PPD 50 μg/ml was chosen at 95 (mean of 99 — 3 SD and 60 + 3 SD)

MI determinations of cells from the 18 Mantoux positive persons on two different days revealed a day to day variation of 1–19 MI units with an average of 7 the difference being independent of the mean MI The two sets of independent determinations were approximately normally distributed and well correlated (r = 0 76) (Table 1)

TABLE 1 *Migration Index Determined on 2 Different Days (MI_I, MI_{II}) in Tuberculin Positive Persons (PPD 50 μg/ml)*

Subject	MI_I	MI_{II}	Subject	MI_I	MI_{II}
1	47	43	10	79	66
2	55	61	11	75	70
3	70	74	12	65	61
4	76	64	13	73	64
5	74	60	14	69	61
6	72	73	15	66	53
7	56	57	16	55	54
8	65	71	17	67	60
9	42	41	18	50	69

The response to particulate antigen was tested by using a suspension of heat killed tubercle bacilli. As seen from Fig 2, cells from 10 tuberculin positive persons were inhibited (mean MI 64) while cells from 4 tuberculin negative persons did not show migration inhibition (mean MI 99) using a concentration of 9×10^7 bacteria/ml cell suspension. The supernatant obtained after centrifugation of the bacterial suspension at 11000 *g* for 10 minutes did not inhibit leucocyte migration of cells from tuberculin positive persons, indicating that no soluble antigen was present in the suspension.

To test the effect of increasing concentrations of PPD cells from 10 tuberculin positive and 6 tuberculin negative persons were migrated against PPD 25 50 and 100 μg/ml. The migration of cells from tuberculin positive persons was inhibited at all concentrations of PPD tested with the mean MI of 79

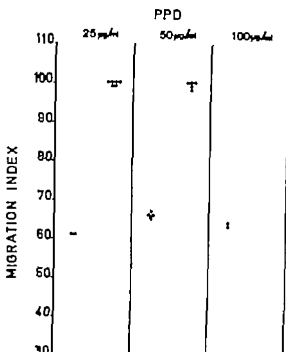


Fig 3 The leucocyte migration test in 10 tuberculin positive ○ and 6 tuberculin negative ● persons, using different concentrations of PPD

(SD 14) 61 (SD 13) and 52 (SD 12) respectively showing that the MI decreases with increasing antigen dose. Paired comparison of the MI at 25 and 50 µg/ml and of the MI at 50 and 100 µg PPD/ml showed significant difference between the groups ($p < 0.01$) The cells from Moro negative children were not inhibited at PPD 25 and µg/ml whereas PPD 100 µg/ml resulted n low MI in 3 out of 6 cases (MI 73 66 and 55) (Fig 3)

To evaluate the migration inhibition of these non-sensitized cells at PPD 100 µg/ml puromycin was added at the concentration of 12.5 and 25 µg/ml. As seen from Fig 4 puromycin prevented the migration inhibition of cells from tuberculin positive persons, while the migration of cells from 6 Moro negative children 3 presenting MI of 65 57 and 44 was uninfluenced by puromycin

Puromycin had little influence on the migration in the absence of antigen—the areas being 5–10 per cent smaller than the corresponding control areas.

As a rule, the migration test was read only after 24 hours incubation. In some cases the result was observed after 48 and 72 hours as well. If PPD 100 and 50 µg/ml was added to tuberculin positive cells the migration inhibition had disappeared after 48 hours, if cells from Moro negative children were inhibited by PPD 100 µg/ml, the inhibition remained also unchanged after 72 hours.

DISCUSSION

Our results obtained by the leucocyte migration test in agarose plates demonstrate that the test functions well with soluble as well as particulate antigen.

Like *Clausen* (6) we found a positive correlation between the intracutane reaction to PPD and the migration inhibition induced by PPD 50 µg/ml. The distinction between the tuberculin positive and the tuberculin negative group was very clear and overlap-

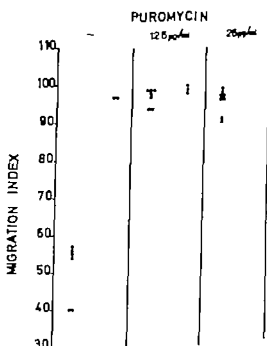


Fig 4 The effect of puromycin 12.5 and 25 µg/ml on the MI of tuberculin positive ○ and negative ● persons tested with PPD 100 µg/ml.

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UPTAKE OF STAPHYLOCOCCAL PROTEIN A BY PERITONEAL MACROPHAGES *IN VITRO*

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Radioactive protein A is actively taken up by rabbit peritoneal macrophages in the presence of immune rabbit IgG most probably due to an immune reaction leaving the Fc sites available for macrophage adherence. The low rate of protein A uptake by guinea pig peritoneal macrophages in the presence of normal or immune guinea pig sera is apparently a result of the extensive Fc-interaction with protein A of the IgG in these sera. Delayed hypersensitivity to protein A of the donor animals does not influence the rate of uptake.

The ability of staphylococcal protein A to interact with the Fc region of IgG molecules (5) confers unique biological properties on this substance (cf. ref 9) Among other activities, isolated protein A is capable of inhibiting phagocytosis of *Staphylococcus aureus* and *Escherichia coli* by human or rabbit polymorphonuclear leucocytes *in vitro* This effect has been supposed to be the result of a competition between protein A and polymorphonuclear phagocytes for active sites on Fc-regions of opsonizing IgG molecules (3)

Independent of the Fc affinity protein A possesses true and distinct antigenic properties as demonstrated by the Fab-reactivity of immune sera (11) and by the induction of delayed hypersensitivity (9) Therefore, protein A is most probably ingested by macrophages which appear to play a role during induction of an immune response (16) These phagocytic cells take up a variety of antigens, which, depending upon their physicochemical state, may be retained at the surface membranes of the macrophages and/or endocytized and catabolized (16)

The aim of this study was to examine *in vitro* the action of peritoneal macrophages, derived from normal or hypersensitive animals, on protein A in the presence of normal or immune sera.

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MATERIALS AND METHODS

Antigens. Protein A was isolated from *Staphylococcus aureus* strain Cowan I according to the procedure described previously (6)

Labelling of protein A by ^{125}I was performed according to method A of the description in (15) A concentration of radioactive iodine of 1 mCi in 0.1 ml was used for 5 mg of protein A.

Radioactivity measurements. Samples were counted in an Electronic counter with an end-window GM-tube detector type Philips 18526. The window thickness was 1.5 mg/cm² A stable background of 10 cpm was obtained. All samples, prepared in duplicate, were measured to more than 1,000 counts at least twice.

Experimental animals. Outbred albino guinea pigs weighing 350–400 g, living in a closed colony and adult albino rabbits, weighing 3–4 kg, of both sexes, were used throughout.

Sensitization of animals. Animals were immunized with a saline solution of 100 µg protein A

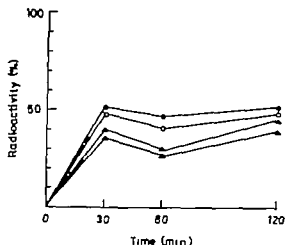


Fig 1 Uptake of protein A by guinea pig macrophages.

- —●— cells from sensitised animals, immune serum present.
- —▲— cells from sensitised animals normal serum present.
- —○— cells from normal animals, immune serum present.
- —△— cells from normal animals, normal serum present.

emulsified in an equal volume of complete Freund's adjuvant (Difco) Volumes of 0.5 ml were injected into both hind footpads. Delayed hypersensitivity to protein A was confirmed 10 days later by intradermal injection of 50 μg protein A in 0 1 ml of isotonic saline. Net swelling of skin at the site of injection was measured after 24 h in guinea pigs and 48 h in rabbits (9)

Preparation of cell suspension. Peritoneal macrophages were obtained by irrigating the peritoneal of the animals with Hanks solution containing 0.05 mg heparin, 3 days after intraperitoneal injection of 3 ml (in guinea pigs) and 10 ml (in rabbits) of 10 per cent proteosepeptone (Difco) in saline. After gentle massage of the abdomen peritoneal fluid was collected over ice. To avoid individual differences pooled peritoneal exudate from 10 animals was used. The cells were centrifuged at 1,000 × *g* in the cold for 10 min and washed three times with chilled Hanks solution. The washed cells were finally adjusted to a concentration of about 3.5×10^7 per ml. The cell preparations consisted of 85–90 per cent macrophages and 10–15 per cent of other cells including lymphocytes, neutrophils, and eosinophils. The viability of cells was measured by the method of *Harris et al.* (8) and found to be higher than 95 per cent in all suspensions.

Sera. Normal sera were pools of sera obtained from non-immunized animals. The immune sera were collected and pooled after immunization twice with 100 μg of protein A in complete Freund's adjuvant (9) The titres of precipitins as tested in double diffusion in agar were 1/64 and 1/128 for guinea pig and rabbit sera, respectively

Uptake of radioactive protein A. Cell suspensions were placed in test tubes, ten per cent of normal or immune serum and the isotope-labelled protein A were added followed by incubation at 37° C with gentle rotation. At zero time and at various time intervals (30 60 and 120 min) some of the tubes were centrifuged for 5 min at 1,000 × *g* in the cold, the supernatant fluid taken off and the cells washed three times with chilled Hanks' solution. The cells supernatants, washings, and control tubes were then counted and the percentage of radioactivity adsorbed to the cells calculated.

RESULTS

The experiments with guinea pig macrophages showed some variations in the rate of uptake of protein A (Fig 1) Apparently the rate of uptake is mainly dependent on the serum used. In the presence of immune guinea pig serum about 50 per cent of total radioactivity was absorbed within 30 min whether the donors of cells had been sensitized to protein A or not. The level of radioactivity was practically invariable during the next 90 min. In the presence of normal guinea pig serum, however both sensitized and normal cells showed a somewhat lower rate of uptake (about 40 per cent) in the first

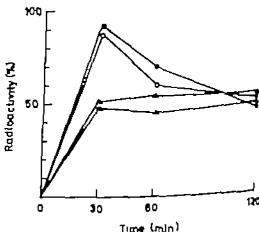


Fig 2 Uptake of protein A by rabbit macrophages. See footnotes for Fig 1

30 min, but after 120 min the amount of radioactive protein A bound to the cells was about 50 per cent.

In the study of uptake of protein A by rabbit macrophages, immune rabbit serum gave a result distinctly different from that of normal serum (Fig. 2). On the other hand, no significant effect connected with the state of activation of macrophages was observed. In the presence of immune serum, approximately 90 per cent of the total radioactivity was bound to either normal or activated macrophages within the first 30 min, the radioactivity of the cells diminishing during the next 90 min to about 50 per cent. Using normal rabbit serum, approximately 50 per cent of the radioactive protein A was bound to the cells within 30 min, this amount being unchanged during the next 90 min. This latter result equals that of control experiments.

DISCUSSION

Protein antigens introduced into the skin of hypersensitive animals are rapidly cleared from the local sites due to ingestion by tissue macrophages (4, 12). In this respect protein A appears to be a normal protein antigen, since it is taken up by macrophages in a short period of time.

Dossett et al. (3) have found that protein A inhibits phagocytosis of bacteria by human and rabbit polymorphonuclear leucocytes due to a competition between these cells and protein A for active sites on Fc of the IgG opsonins. Incubation of protein A alone with leucocytes did not alter the phagocytic properties of the cells against bacteria. In our experiments also the variation in the rate of phagocytosis, or more precisely macropinocytosis (2), of protein A IgG complexes by macrophages is most probably due to an unspecific reaction of protein A with Fc sites. The observed difference between rabbit and guinea pig sera fits well with the difference in Fc-reactivity against protein A of IgG from these sera (7).

Protein A together with immune rabbit IgG was taken up much more extensively than in the presence of normal rabbit and normal and immune guinea pig IgG. This is in agreement with a Fab-reaction between protein A and immune rabbit IgG (11), the resulting complex being bound to the macrophage membrane via the free Fc region. Cells of the mononuclear phagocytic system possess specific receptors which require the Fc site of IgG (1). Moreover material firmly bound to the macrophage membrane is taken up to a greater extent in terms of its extracellular concentration, while materials which are not adequately bound to the surface of the macrophages are taken up at a rate which is directly proportional to the extracellular concentration (2). This may explain the lower rate of protein A uptake in the presence of normal rabbit serum, with a small protein A IgG interaction, and normal guinea pig serum with approximately 100 per cent Fc reactivity of the IgG molecules (7). In both cases the final uptake equals that of the controls. The somewhat higher rate of uptake in the presence of immune than of normal guinea pig serum is in accordance with a competition between Fab and Fc sites of immune IgG for protein A, leaving some protein A IgG complexes with free Fc sites available for macrophage adherence.

In the experiments of *Dossett et al.* (3) complexes of protein A and IgG opsonins most probably had not been taken up by phagocytic cells since macropinocytosis—the most prominent action of macrophages (2)—has never been demonstrated in polymorphonuclear leucocytes (10).

It is of interest that the radioactivity of the cells in the experiments including immune rabbit serum diminished after 60 min to a "normal" level of about 50 per cent (Fig. 2). It is possible that immune complexes firmly bound to the macrophage membrane are metabolized more rapidly than free molecules.

In our study the activation of the cells by the evoking of a state of delayed hypersensitivity to the antigen in donor animals had no demonstrable influence on the rate of

uptake. *Rhodes* & *Lind* (13 14) also concluded that antigens are ingested by peri toneal macrophages *in vivo* regardless of the immunological state of the animals.

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IN VITRO TRANSFORMATION OF MOUSE SPLEEN CELLS BY CASEIN, PHYTOHAEMAGGLUTININ AND ALLOGENEIC CELLS IN CASEIN-INDUCED AMYLOIDOSIS

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Spleen cells from casein-treated mice developing amyloid were tested for *in vitro* reaction to phytohaemagglutinin (PHA) allogeneic lymphocytes (MLC) and casein, using uptake of ^{14}C-thymidin as a measure of the transformation. The response to PHA and casein was found to be unaffected by amyloid development, whereas the MLC was depressed in amyloid mice. Our results indicate 1) that amyloid development may be accompanied by elective depression of a certain part of the function of immune cells, 2) that tolerance to the applied casein is not a pre-requisite for amyloid development.

Cathcart et al. (1971) using the *in vitro* macrophage migration test, showed that casein-treated guinea pigs developed a cellular immune response to casein that waned when the animals became amyloidotic. Cellular immune responsiveness to other antigens was unaffected. *Brecetti et al.* (1972) found a significant depression of the *in vitro* responsiveness of circulating lymphocytes to casein in pre-amyloidotic and amyloidotic guinea pigs. This lead the authors to the hypothesis that tolerance to the immunogen might play an important role in the pathogenesis of casein-induced amyloidosis.

In the present study this hypothesis was re-examined by means of *in vitro* stimulation of spleen cell cultures from mice in different stages of amyloidosis.

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MATERIAL AND METHODS

Experimental Animals

Randomized female C3H-mice, 6–8 weeks old at the start of treatment, were derived from inbred stocks maintained in the Institute of Medical Microbiology Copenhagen. In each of 6 separate experiments, spleen-cell suspensions from a series of 9 mice were cultured. The mice were given 6 weekly subcutaneous injections of 0.5 ml 5 per cent casein (Ble & Berntsen) in 0.25 M NaOH for 0, 1 2, 3 4, 5 6, 8 and 10 weeks, respectively. Stimulating cells in the mixed lymphocyte cultures (MLC) were derived from spleens of female F_1 hybrid mice of inbred male Balb/C- and female C3H-mice, H2-locus d and b, respectively (*Staats* 1972) Three mice died during the treatment.

Histological Examination

Sections of spleen, liver and kidney were stained with methyl green-pyronine, PAS and alkaline Congo red. Amyloid was identified by its morpho-

TABLE 1 *Spleen Amyloid in Casein-treated C3H Mice*

Duration of casein treatment (weeks)	0	1	2	3	4	5	6	8	10
Number of mice	5	6	6	6	6	5	6	5	5
Spleen amyloid mean	0	0	1 7	3.2	4.3	4.6	5.2	4 8	5 2
(range)	(0)	(0)	(1–2)	(3–4)	(4–5)	(4–5)	(5–6)	(4–5)	(5–6)

logy and by its green dicroism with Congo red under crossed polars. The degrees of spleen amyloidosis were graded according to the scedule of *Christensen & Hjort* (1959)

Tissue Culture Medium

To medium RPMI 1640 (Flow laboratories) was added L. *glutamin* 2 mM, penicillin and streptomycin 50 i.u./ml of each, heparin sodium U.S.P (Novo) 10 i.u./ml, Hepes buffer 20 mM and the medium was supplemented with 5 per cent of inactivated human serum (*Adler et al.* 1970)

Cell Preparation

The mice were killed by cervical dislocation. The spleens were minced with knives and filtered through four layers of gauze into complete tissue culture medium. The cell suspensions were washed twice in this medium and adjusted to 2 × 10^6 mononuclear cells per ml.

Cell Cultures

The cell suspensions were dispensed in ½ ml aliquots, containing 10^6 responding cells per ml. Stimulated cultures further contained reconstituted phytohaemagglutinin-P (PHA) 25 µl/ml (Difco) casein (prepared from the above injection-liquid) 2 mg/ml or α-casein (Behringwerke) 0.7 mg/ml To MLC's were added 10^6 stimulating (Balb/C × C3H)F mononuclear spleen cells per ml.

Each cell suspension was cultured in triplicate at 37 C in tightly capped polyethylene tubes and

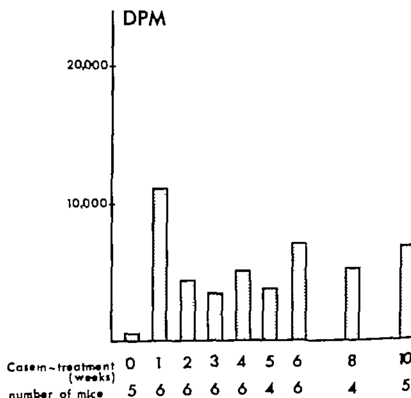


Fig 1 Unstimulated cultures. 20 hours incubation. Ordinate disintegrations per minute, mean of indicated numbers of experiments.

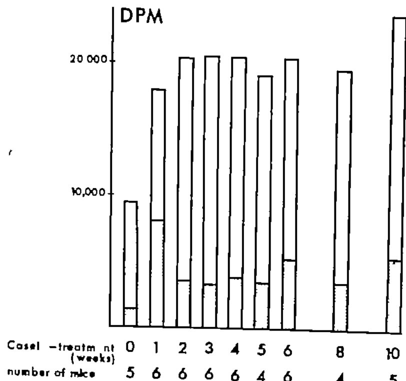


Fig 2 PHA-stimulated cultures (whole bars) Unstimulated cultures (hatched areas) 44 hours' incubation. Ordinate disintegrations per minute, mean of indicated numbers of experiments.

terminated late in the exponential phase of growth (determined by preliminary experiments) Cultures stimulated by PHA and casein-preparations were harvested after 44 hours, and MLC after incubation for 68 hours. Unstimulated cultures were terminated after incubation for 20 44 and 68 hours. Twenty hours before harvest, 0 04 μCi [14]C-thymidin (The Radiochemical Center Amersham, England) was added to each tube. The cells were harvested on filters and the activity counted by liquid scintillation. The results were expressed as the means of triplicate samples in disintegrations per minute (DPM) per culture.

RESULTS

Histological Examination

Mice treated for 1 week exhibited an intense splenic pyroninophilia but amyloidosis did never occur Animals treated for 2 weeks showed initial spleen deposits of amyloid. A 4–5 degree amyloidosis of all the spleens and small amyloid deposits of livers and kidneys were present by 4 weeks and thereafter (Table 1)

Unstimulated Cultures

The activity of cells from casein-treated mice always exceeded that of cells from un treated mice, indicating that a cellular immune reaction was caused by the treatment (Fig. 1–5) The greatest background activity (expressed by unstimulated cultures incubat ed for 20 hours—Fig. 1) was seen in cultures derived from mice treated for 1 week. Cul tures from these mice showed, after incuba tion for 2 and 3 days, a continuous fall in activity (Fig. 1 2, 3) while further treatment resulted in an increased activity if cultures were incubated for periods covering from 2 to 3 days. Treatment covering from 1 to 2 weeks resulted in a fall in background activity (Fig 1) No further tendency to decreased

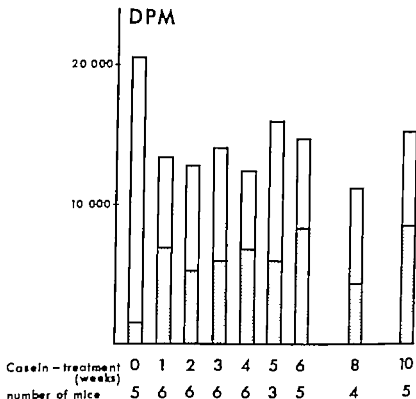


Fig. 3 MLC-stimulated cultures (whole bars) Unstimulated cultures (hatched areas) 68 hours' incubation. Ordinate disintegrations per minute mean of indicated numbers of experiments.

activity was found if treatment covered from 3 to 10 weeks.

PHA stimulated Cultures

The PHA induced activity of the spleen cell cultures (Fig 2) did not decrease in either strain during the casein treatment, always exceeding the activity measured in cultures from untreated mice

MLC stimulated Cultures

Fig 3 shows that the *in vitro* response of spleen cells to allogenic cells in treated mice was inferior to that in untreated mice. The MLC-response was reduced already in the non-amyloidotic group treated with casein for 1 week and did not further decrease when amyloidosis occurred. The difference in the MLC response in untreated and amyloid mice was significant ($p<0.01$ Mann-Whitney rank sum test) whether the difference (stimulated unstimulated activity) or the stimulation index (stimulated/unstimulated activity) was employed.

Casein stimulated Cultures

The employed solution of whole-casein exerted an inhibiting action on the cell-growth the activity of unstimulated cultures being greater than that of stimulated cultures in untreated mice (Fig 4) Although the increase in activity of stimulated cultures was rather small, Fig 4 shows that a cellular immune response exceeding this inhibition, had developed in spleen cells of all casein-treated mice. There was no sign of altered activity parallel to the development of amyloidosis.

The *in vitro* response to α-casein was tested only in 4 series of mice Fig 5 indicates that this casein-fraction had unspecific mitogenic properties, stimulating also cells of untreated mice There was no reduction of the response in amyloidotic mice.

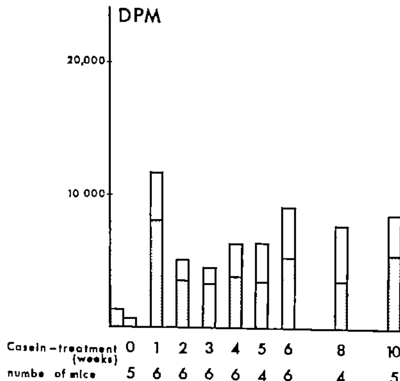


Fig 4 Casein-stimulated cultures (whole bars) Unstimulated cultures (hatched areas) 44 hours incubation. Ordinate disintegrations per minute mean of indicated numbers of experiments.

Variability of the Culture System

The coefficient of variation (C.V) for tripledeterminations of each culture-combination for unstimulated cultures, incubated for 20 44 and 68 hours, was 13 per cent, 4 per cent and 12 per cent, respectively for PHA stimulated cultures 7 per cent, for casein and α-casein stimulated cultures 6 per cent and 7 per cent, respectively and for MLC 9 per cent.

The interexperimental variation of unstimulated cultures was C.V = 46–61 per cent. For PHA-stimulated cultures the C.V was 42 per cent, for casein- and α-casein-stimulated cultures 45 per cent and 29 per cent respectively and for MLC's 58 per cent.

DISCUSSION

It appears from the present tests of the *in vitro* reactivity of thymus-derived lymphocytes (*Festenstein et al* 1969) *Janossy & Greaves* (1971) that a general depression of the cellular immune reactivity does not develop during amyloidogenesis since the PHA response remains unaffected. This was also found by *Briccetti et al.* (1972) The cellular immune reactivity measured by the MLC, however was found to be depressed in pre amyloid and amyloid mice. Also the immune functions measured by graft rejection (*Ranløv & Jensen* 1966) and graft-versus-host reaction (*Hardt & Claesson* 1971) have been found to be depressed in amyloid mice. The relative importance if any of the various subpopulations of leucocytes (*Lanotte & Pasujel* 1973) for amyloid development in casein-treated mice is not known. The seemingly contradictory results obtained by different test systems therefore may reflect a discriminatory influence of casein treatment on the different cell systems. As regards the results

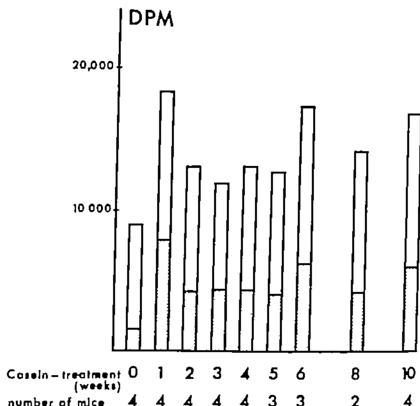


Fig 5 α-casein-stimulated cultures (whole bars) Unstimulated cultures (hatched areas) 44 hours' incubation. Ordinate: disintegrations per minute mean of indicated numbers of experiments.

obtained by our tests the lacking evidence of emergence of a specific unresponsiveness to casein in casein-treated mice developing amyloid, is strongly supported by the inability of grafted unsensitized syngeneic lymphoid cells to abrogate amyloid development in casein-treated mice (*Hardt et al.* 1972) Our results, indicating an unimpeded reactivity of spleen cells to casein, is at variance with the inhibited reactivity to casein of circulating leucocytes (*Briccotti et al.* 1972) as well as at macrophages (*Cathcart et al.* 1971) harvested from casein-treated guinea pigs developing amyloid. Immunologic tolerance to casein therefore may well develop in casein-treated guinea pigs, but as judged from our results with mice it is not a prerequisite for amyloid development. Our casein solution, however contains more than one antigen fraction (*Ebbesen* 1971) The possibility therefore exists that an amyloid-inducing fraction may differ from fraction(s) eliciting the reactions measured by the immune tests employed here.

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BRIEF REPORTS

THE CAPILLARY TUBE LEUCOCYTE MIGRATION TECHNIQUE (LMT) AS A METHOD FOR DETECTION OF SERUM INHIBITING ACTIVITY OF TUMOUR DIRECTED CELLULAR HYPERSENSITIVITY IN PATIENTS WITH RENAL CARCINOMAS

Mogens Kjær

Serum inhibiting activity of tumour-directed, cellular hypersensitivity could be demonstrated in si out of seven patients with renal carcinomas by incubating preoperative serum with hypernephroma extract and leucocytes in autologous or allogeneic combinations in the capillary tube leucocyte migration technique.

Factors capable of blocking tumour-directed, cellular hypersensitivity *in vitro* have been demonstrated in serum from patients with an actively growing neoplasm. These studies have been carried out mainly using the colony inhibition technique (6 7) microassay target cell destruction (2, 9) and the lymphocyte transformation test (13).

Animal experiments have indicated that the macrophage migration inhibition technique could be used for demonstration of serum blocking activity in mice bearing primary Moloney virus-induced tumours, transplanted, chemically induced tumours (5) and adenovirus 12-induced CBA tumours (10).

According to *Currie* (1973) at least two effects of serum on tumour-directed, cellular hypersensitivity must be considered separately:

1. Blocking, i. e. effects obtained by pre-incubation of the test serum with the target cells. Affinity for target cells.
2. Inhibition, i. e. effects obtained by including the test serum in the lymphocyte suspension or in the test system. Affinity for lymphocytes.

In the present study preliminary results regarding demonstration of inhibiting activity of tumour directed, cellular hypersensitivity in serum of patients with renal carcinomas using the LMT are reported.

Material and Methods

The series comprised 7 consecutive patients with hypernephroma without evidence of distant metastases. As control patients, a comparable number of persons, matched as regards age and sex, without evidence of benign or malignant tumours, hypertension or auto-immune diseases were tested following the same protocol as for the cancer patients.

After removal of the kidney a portion of tumour and of the renal tissue not macroscopically invaded by tumour was obtained. These specimens were cut separately into small pieces, suspended in Hanks solution, and homogenized in a M.S.E. homogenizer for 8 min. The homogenates were kept at 4° C overnight and then centrifuged at 1000 g for 20 min. The protein concentration of the supernatant was adjusted to 1 mg per ml by dilution with Hanks' solution and lyophilized.

The LMT was performed as described by *Bendixen & Søborg* (1969). In systematic extract titration experiments (8) the highest concentration of extracts not causing non-specific stimulation or inhibition of migration of normal leucocytes from 29 control patients was determined to be 400 μg protein per ml medium, and this dose was used throughout.

On the day of the test, blood was collected in heparin and Dextran. After sedimentation, the leucocytes were washed 4 times in Hanks' solution and collected in capillary tubes. Each of these was placed in a 1.0 ml culture chamber containing TC 199 with 10 per cent horse serum. To the test cultures, 0.4 ml of tissue extract containing 400 μg of protein was added. All cultures were set up in quadruplicate. After 24 hours, the migration area of leucocytes around the opening of the capillary tube was measured in a projection microscope. Within a set of identical cultures, the variation

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from one migration to another did not exceed 10 per cent. The average migration area of extract containing (M_X) cultures and control (M_O) cultures determines the migration index (MI) MI = M_X/M_O.

TABLE 1. *Inhibiting Effect of Pre-operative Serum on the Tumour-directed Cellular Hypersensitivity in Patients with Renal Carcinoma. Complete Research Protocol Applying to a Patient with Hypernephroma*

*Patient**	
Culture chamber	*MI*
1 autologous serum 0.05 ml	0.98
2 allogeneic serum 0.05 ml‡	1.03
3 autologous tumour	0.78
4 autologous renal tissue	1.11
5 autologous tumour + autologous serum 0.05 ml	1.23
6 autologous renal tissue + autologous serum 0.05 ml	0.84
7 autologous tumour + allogeneic serum 0.05 ml	0.77
8 autologous renal tissue + allogeneic serum 0.05 ml	0.85

Control patient†	
Culture chamber	*MI*
1 autologous serum 0.05 ml	0.90
2 allogeneic serum 0.05 ml$	1.06
3 allogeneic tumour	1.12
4 allogeneic renal tissue	1.05
5 allogeneic tumour + autologous serum 0.05 ml	1.08
6 allogeneic renal tissue + autologous serum 0.05 ml	1.01
7 allogeneic tumour + allogeneic serum 0.05 ml	1.13
8 allogeneic renal tissue + allogeneic serum 0.05 ml	1.09

* Hypernephroma patient. Test performed on Sept. 10. Nephrectomy performed on Aug. 16. Serum sample drawn on Aug. 14. Antigen autologous tumour and renal tissue extract, concentration 400 μg per ml respectively.

† Control patient with duodenal ulcer. Resection according to Billroth II performed on Sept. 18. Serum sample drawn on the day of the test, Sept. 10.

‡ Allogeneic serum—serum from the control patient.

$ Allogeneic serum and tumour—serum and tumour from the patient with hypernephroma.

Fig. 1. Inhibiting effect of pre-operative serum on tumour-directed, cellular hypersensitivity in patients with renal carcinomas.
o———o – autologous tumour extract and autologous serum.
o- - - - -o – allogeneic hypernephroma extract and allogeneic serum from a patient with hypernephroma.
o— — —o – allogeneic hypernephroma extract and autologous serum.
Column A) Migration indices without serum added to the culture chamber.
Column B): Migration indices after addition of 0.05 ml of decomplemented serum to the culture chamber containing 1.0 ml.

A MI of less than 1.0 indicates an inhibition, a value of more than 1.0 indicates a stimulation of leucocyte migration. The normal range x ± 2 S.D. of the MI's in the control group at a concentration of tissue extract of 400 μg per ml, was 0.79–1.19. In serum inhibiting experiments, the LMIT was set up as described above. However a number of culture chambers were included which contained leucocytes, tumour extract, and serum in autologous or allogeneic combinations. Comparable samples of serum from control patients were included in the protocol. If serum was not used immediately it was stored at −20° C until the test was performed. Immediately before use, the serum samples were decomplemented by heating at 56° C for 30 min. The volume of serum in the chambers was arbitrarily chosen, 0.05 ml per ml. No serum titration experiments were performed in this study. A complete protocol for one of the patients and the matched control patient in the series is shown in Table 1.

Results

Three out of seven patients with renal carcinomas had a stimulation (MI > 1.19) and three

out of seven inhibition of leucocyte migration (MI < 0.79) towards autologous or allogeneic hypernephroma extract before addition of serum (Fig 1 column A) According to *Søborg* (1968) on contact with specific antigen stimulation as well as inhibition of leucocyte migration are indicative of a cell mediated hypersensitivity reaction. No reactivity towards normal, adult renal tissue extract was found (Table 1)

After addition of 0 05 ml of autologous or allogeneic serum from patients with hypernephroma, six out of seven patients had MI s within the normal range and one out of seven slight stimulation (Fig 1 column B)

Serum from control patients did not affect the MI. Likewise serum from hypernephroma patients did not affect the migration of control leucocytes (Table 1)

Discussion

The finding that pre-operative serum from patients with hypernephroma was capable of preventing tumour extract-induced stimulation or inhibition of leucocyte migration in these patients indicates the presence of inhibiting activity in the serum samples tested.

The nature of this activity is not clearly understood but at least two mechanisms are possible

1. Tumour-associated transplantation antigens present in the tumour extracts could be masked by antigen-antibody complexes (11) thus preventing them from inducing leucocyte migration stimulation or inhibition.
2. Lymphocytes could be coated by circulating tumour-associated transplantation antigens (4)

Preliminary experiments indicates that repeated washing of peripheral leucocytes from patients with disseminated hypernephroma removes a factor that inhibits their reaction in the presence of tumour extract. This factor might be tumour antigen coating the cells.

In view of the important implications on the evaluation of the clinical stage and prognosis of cancer patients, it must be considered a major goal to develop simple and rapid *in vitro* tests for the detection of serum inhibiting activity in man. The results obtained in the present study represent a preliminary attempt in this direction.

The study was supported by *The Danish Cancer Society*

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THE ISOLATION OF AN AGENT RELATED TO UUKUNIEMI VIRUS FROM NORWEGIAN IXODES RICINUS TICKS

Terje Traavik, Reidar Mehl and Eva Mood Pettersen

Although the existence of tick-borne arboviruses has been demonstrated many years ago in Denmark (3) Finland (5) and Sweden (6) no isolates have ever been reported from Norway

Clinical, epidemiological and ecological observations have, however indicated the presence of arbovirus foci in this country (1 7 8) In addition, a recently published serological investigation demonstrated that the frequency of cattle seropositive for tick-borne encephalitis (TBE) virus was relatively high in some areas along the Norwegian coast (9) These results encouraged further investigations in the field.

Early in May 1973 8 engorged *Ixodes ricinus* nymphs were collected from migrant passerine birds (species unknown) captured at Store Færder a small island in the Oslofjord. The ticks were transported alive to the laboratory and were immediately processed for irus isolation experiments. After rinsing in saline, they were ground in a mortar in PBS pH 7 4 with the addition of mycostatin, penicillin and streptomycin. The suspension was clarified by moderate centrifugation and inoculated intracerebrally into 1 day old laboratory mice. Eleven days later the mice were moribund; the brains were removed, homogenated in PBS with antibiotics and passed into new litter of newborn mice which were moribund 7 days later

In the further passages, incubation periods were reduced to 3–4 days. Using brain material from the 4th mouse passage, it was demonstrated that the isolated agent readily passed 220 as well as 100 nm millipore filters. By electronmicroscopy approx. 80 nm spherical particles were demonstrated in the filtrates

Brain material from the 5th mouse passage was processed for haemagglutinating activity by the acetone-ether extraction (AE) sucrose-acetone extraction (SA) and alkaline aqueous suspension methods of *Clarke & Casals* (2) employing chicken erythrocytes. The acetone-ether extract and the alkaline aqueous suspension showed no haemagglutinating activity within the pH range of 5 75–7 4 when incubated at +4 22 and 37 C. After Tween-ether treatment according to *Mussgay & Rott* (4) low grade activity was detected at 37 C. The sucrose-acetone extract produced clear cut haemagglutination patterns at 22° and 37 C, the highest titre being obtained at 37 C and an optimum pH value of 5.75

The SA antigen preparation was utilized in a haemagglutination inhibition (HAI) test, employing micro-titration equipement (Cooke Engineering Co, Alexandria, Virginia) but otherwise performed as described by *Clarke & Casals* (2) Antibody preparations, immune mouse serum and mouse immune ascitic fluids had been obtained from the Yale Arbovirus Research Unit.

Eight haemagglutinating units of antigen were used in the final test.

The following specific antibody preparations were utilized

RSSE (Sophy) mouse immune ascitic fluid FD	6/31/66
Tribec mouse immune ascitic fluid FD	9046 11/20/67
Eastern Equine Encephalitis ascitic fluid FD	46729 11/30/66
Western Equine Encephalitis ascitic fluid FD	10/3/66
Uukuniemi (S-23) immune mouse serum FD	12/15/71

The Uukuniemi antibody preparation demonstrated haemagglutination inhibition at a dilution of 1/80, while the other preparations showed no inhibiting effect at all

It is concluded that an agent serologically related to the Uukuniemi group (Bunyamwera super group) of arboviruses has been isolated.

This represents the first isolation of an arbovirus in Norway As nothing is known concerning

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the movements of the host birds in the days prior to their capture neither the geographical origin of the isolated agent nor the tick vectors carrying it can be definitely determined.

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TRANSPLACENTAL TRANSMISSION OF *NOSEMA CUNICULI* IN THE BLUE FOX (*ALOPEX LAGOPUS*)

S. F. Mohn, K. Nordstoga, J. Krogsrud and A. Helgebostad

Nosematosis (encephalitozoonosis) is a world-wide protozoan disease which is best known in laboratory rodents where the infection is usually inapparent or runs a mild course. Some discrepancy exists as to whether the correct name of the causative organism should be *Nosema cuniculi* or *Enc phalitozoon cuniculi* (2, 4 8 13) In this paper the terms *N sema cuniculi* and nosematosis are used, without however preferring one view or the other

More rarely nosematosis has also been reported in species other than rodents, including man (1 3 5-7 9 11 12) During recent years, this infection has been a major problem, and has caused heavy losses, in the breeding of blue foxes in the Nordic countries. This observation probably indicates that the *N sema* organisms are more pathogenic in blue foxes than in other mammalia. Only young growing cubs in the first few months of their life are affected, and as in other animals the main lesions are found in the central nervous system and the kidneys. A unique finding is that nearly all affected foxes display vascular lesions similar to polyarteritis nodosa (7) An evident hyper gammaglobulinaemia is a constant finding in severely affected animals (to be published)

The natural mode of transmission is mostly unknown, and although vertical transmission has been suggested by several workers and congenital infection has been reported in gnotobiotic rabbits (2) the present authors are unawar of any pre ious experimental data confirming this belief.

The strain of N sema used in this experiment was isolated from a spontaneously infected fox, the organism was propagated in the abdominal cavity of Swiss albino mouse (strain VMRI/Bom) and in cultures of canine kidney cells and ovine chorioid plexus cells (10) Cell cultures were easily infected by inocula from mouse peritoneal exudate and the organism was also readily subpassaged in the cell cultures F rther investigations have shown that the agent may be frozen and stored at liquid N_2-temperature.

The transmission experiment was carried out at the Research Station for Fur-bearing Animals, Heggedal, where nosematosis had never occurred. Three vixens were infected during the gestation period. One of the animals did not conceive, and one died during the delivery in association with obstetric complications. The 3rd animal, which was fed 4 parasitized mice and 3.5 ml cell culture fluid, mixed with the feed, 8 days after mating, gave birth to 9 pups which all successi ely developed clinical signs indicative of nosematosis. The first symptoms were observed when the cubs were approximately 3 weeks old. At autopsy all the animals showed advanced lesions characteristic of nosematosis, including polyarteritis nodosa. Urine specimens from the cubs which either died or were killed in a moribund condition were obtained at autopsy all samples contained excessive numbers of individual spores characteristic of *Nosema* organisms, i.e. of elongated shape and possessing one or two polar vacuoles. Numerous similar parasitic structures were seen in sections from most organs. Electron microscopic investiga tion has confirmed their identity Urine specimens and organ suspensions from the cubs were injected into the abdominal cavity of mice and *Nosema* organisms were reisolated in all cases.

As the mothers of the cubs affected with nosematosis always remain healthy and parasites have never been demonstrated in the organs of adult foxes from farms where nosematosis is widely distributed among the pups, it is most probable that the offspring were infected in utero in this experiment, after oral infection of the mother It seems likely that vertical transmission is also most common in field cases, although other routes of transmission cannot be excluded.

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CONTAMINATION OF SWEDISH CEREALS WITH OCHRATOXIN A

P Krogh B Hald P Englund L. Rutqvist and O Swahn

Ochratoxin A, a dihydro-isocoumarin derivative linked through its 7-carboxy group to L-β-phenylalanine (5) is a secondary metabolite of several species included in the fungal genera *Aspergillus* and *Penicillium* (2) This mycotoxin exhibits the toxicity primarily through a damage to the proximal tubuli (8) and has been observed in causal relation to a naturally occurring renal disease, porcine nephropathy (5) Ochratoxin A has been found as a natural contaminant of maize and barley in U.S.A. (6 11 12, 13) at levels from 12 to 166 μg/kg. In Canada the mycotoxin was encountered in wheat, oats, barley rye, peanuts, and white beans at levels from 20 μg/kg to 27,000 μg/kg (9 10)

In Denmark ochratoxin A has been detected in horse beans (4) and was found in quantities (up to 27,500 μg/kg) in barley and oats associated with field outbreaks of mycotoxic porcine nephropathy (5) Because of the close geographical position it was found reasonable to conduct a similar screening of cereals produced in Sweden.

Material and methods

Samples of cereals (barley and oats) used as animal feed were collected by random during spring 197? from 84 farms situated in central and southern Sweden. The water content (14) was measured at harvest, when the lots were transported to the storage at the farms. Germination (1) was determined after the cereals had been kept in storage for 6–8 months. Conservation (drying ventilation) was carried out in some farms, whereas other farms stored the cereals without any treatment. Ochratoxin analysis was carried out as a TLC method (7) involving extraction with water chloroform, purification of the extract on an aqueous sodium bicarbonate-diatomaceous earth column, and thin-layer chromatography by use of densitometer techniques. The presence of ochratoxin from samples that appeared to be positive by TLC was confirmed by derivative formation.

TABLE 1 *Cereal Samples Containing Ochratoxin A. (Total Number Assayed 84)*

Sample No.	Commodity	Water content at harvest per cent	Germination per cent	Concentration of ochratoxin A μg/kg
A 363	barley	16.7	99	86.7
A 489	barley	26.4	85	16 0
A 332	barley	23 4	60	53.2
A 378	barley	37 5	0	139.5
A 380	barley	23.1	66	409.5
A 374	oats	31 4	31	76.5
A 379	oats	28.2	20	28.8

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Results and discussion

Seven cereal samples out of 84 (8 per cent) contained ochratoxin A, and the concentration in these samples is given in Table 1 Ochratoxin

B, C and esters were not detected. The water concentration at harvest of the contaminated samples was fairly high, but no direct correlation between ochratoxin concentration and water concentration appears. The same lack of correlation seems to exist concerning ochratoxin concentration and decrease of germination (Table 1) In the Danish screening study 58 per cent of the samples contained ochratoxin A (3) However these samples were not collected by random, but according to a sampling plan based upon epidemiological information on recent cases of mycotoxic porcine nephropathy Therefore the samples were suspected to be contaminated.

Etiological investigations in Denmark have pointed to ochratoxin A as a major determinant of mycotoxic porcine nephropathy (3) As barley and oats are used as pig feed in Sweden as well, it seems reasonable based upon the results of the present investigation, to speculate whether mycotoxic porcine nephropathy occurs in Sweden as well. Epidemiological studies are in progress in order to elucidate this possibility

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IS THE INCIDENCE OF *YERSINIA ENTEROCOLITICA* INFECTION INCREASING?

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Since a small outbreak of infection with *Yersinia enterocolitica* in our hospital (6) increased numbers of sera with high antibody titres to *Yersinia* have been observed in our laboratory. This might be due to the increased interest and to improved diagnostics, but it also may reflect a true rise in the incidence of *Yersinia* infection.

Therefore, a comparative study on the sera collected during the years 1969–1971 and 1973 was carried out. The blood samples from the years 1969–1971 were from 197 putatively healthy mothers in a maternity hospital and from 50 blood donors. The sera had been stored at −20°C. The material of 1973 includes 101 mothers in a maternity hospital, 542 blood donors, and 258 staff members in wards and in a laboratory. 257 of the blood donors in 1973 were from Oulu in the northern Finland, all other samples were collected from people living in the southwestern part of the country. The sera were titrated in two-fold dilutions with OH antigen of *Y. enterocolitica* serotype 3 and 0 antigens of *Y. enterocolitica* serotype 9 and of *Y. pseudotuberculosis* IA as described previously (6).

The results are given in Table 1 (titres are expressed as reciprocals). Frequency of antibodies in the 1969–1971 samples is of the same magnitude as that reported by *Ahvonen* for samples collected in 1968–1970 (1). The samples from 1973 show an about ten-fold higher frequency of high titre ($\geq$ 160) *Yersinia* antibodies than the samples collected during the years 1969–1971 ($P < 0.001$; $\chi^2 = 19.0$ for 1 d.f.). The increase is mainly due to the occurrence of antibodies against *Y. enterocolitica* serotype 3 and serotype 9, high titre antibodies against *Y. pseudotuberculosis* IA were observed in 1.0 per cent of the samples collected on 1973. The low frequency of high titres in the older samples can hardly be attributed to the storage, since low titre antibodies were found to the same extent in the 1973 samples as in the old ones. The low titres include nonspecific ones, whereas titres $\geq$ 160 can be considered an indication of recent *Yersinia* infection (1). For the 1973 there was no difference between the samples originating in the northern or southwestern parts of Finland. Likewise, antibody frequencies were about equal among blood donors, hospital patients and staff members.

TABLE 1 *Prevalence of Yersinia Antibodies in Blood Samples Collected in 1969–71 and 1973*

Year of collection	Number of sera studied	Titre	Per cent of sera with antibodies to			
			Y. enterocolitica serotype 3	*Y. enterocolitica* serotype 9	*Y. pseudotuberculosis* IA	Total
1969–71	247	20–80	4.9	9.3	1.2	15.4
		≥ 160	0.8	–	–	0.8
1973	901	20–80	5.9	9.6	1.1	16.6
		≥ 160	5.7	2.2	1.0	8.9

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These findings indicate a roughly ten-fold rise over a period of 2–4 years in the prevalence of *Yersinia* infection in Finland. Our own clinical ex-

perience points to the same direction. Accidentally our suspicions have drastically been confirmed by a current epidemics of a *Y. enterocolitica* infection in a garrison in the southern Finland.

Whatever the first origin of the infection may be, a spread is easily understandable. Our earlier observations (6) indicate the high infectivity of the organism and transmittance of the infection from person to person with an incubation period of about 10 days. An additional factor enhancing the spread is that many cases present a variety of minor symptoms (2, 6) that are easily overlooked by the patient and doctors. The symptoms may often resemble those of appendicitis, gynecological disorders or rheumatoid arthritis. Also *Y. enterocolitica* has proven a resistant micro-organism: it survives for several weeks in tap water at 4 C (*Olkkonen* unpublished) and grows at room temperature (5). Recent findings reported from the North America (3, 4) suggest that a same kind of increase in the prevalence of *Y. enterocolitica* infection, as described here, may be occurring also elsewhere than in Finland.

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NO EVIDENCE FOR A CARBOHYDRATE MOIETY AFFECTING THE CLEARANCE OF CIRCULATING HUMAN LEUKOCYTE INTERFERON IN RABBITS

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After treatment with neuraminidase, human leukocyte interferon (HLI) retained its characteristic heterogeneity when subjected to isoelectric focusing. The clearance of HLI from the circulation of rabbits, injected intravenously was unaffected by treatment with neuraminidase or subsequent treatment with galactose oxidase. Complementary chemical treatments were similarly without effect. None of the treatments resulted in destruction of biological activity. Sialic acid and galactose or its derivatives do not appear to contribute to either the antiviral activity or the pharmaco-kinetics of HLI.

Factors that influence the pharmaco-kinetics of interferon are little known (10) Human leukocyte interferon (HLI) is presently used for clinical trials (14 25) and a model for studying its pharmaco-kinetics in rabbits has been established (3) Interferon is cleared rapidly from the blood after intravenous injection and dose schedules that increase the persistence of serum interferon are actively investigated (18, 3) An alternative approach lies in the possible modification of the interferon molecule. It has been suggested several times (9) that interferon may contain a carbohydrate moiety and *Schonne et al.* (20) reported that treatment with neuraminidase greatly affected the isoelectric points of rabbit interferon. The presence of sialic residues in variable amounts could account for the considerable heterogeneity of interferons with respect to charge (7 23) Recently *Dorner et al.* (6) presented evidence that rabbit interferon contained terminal sialic acids with penultimate galactose residues, presumed to link through carbohydrate to a polypeptide chain. Such an arrangement has been demonstrated for many glycoproteins (21) *Morell et al.* (15) have shown that the clearance of many such glycoproteins from the blood is increased by the exposure of the penultimate galactose and can be decreased again by the re introduction of terminal sialic acid (or artificially by the oxidation of the galactose residues) This phenomenon is seen as a factor controlling the level of circulating glycoproteins.

We report here on the possible contribution of terminal sialic acid and penultimate residues, consisting of galactose, galactosamine or acetylgalactosamine (2) towards the clearance of HLI from the circulation of rabbits.

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Both chemical and enzymic treatments are used to either remove or destroy galactose and sialic acid and the effects of such treatments on the antiviral activity isoelectric points and pharmaco-kinetics of *HLI* are followed.

MATERIALS AND METHODS

Production and Purification of Interferon

HLI was prepared from human leukocytes treated with Sendai virus (24) in the presence of human serum from which most of the globulins had been removed (5) Crude interferon was concentrated some 50 fold, by precipitation with acid potassium thiocyanate, to give a preparation containing 600,000–1,000,000 units/ml and ca 50 mg/ml of protein (5) HLI was partially purified by the selective precipitation of inert proteins from ethanolic solution with rising pH, to a specific activity of ca. 1 000 000 units/mg of protein (4)

Assay of Interferon

HLI was assayed by the reduction of Vesicular Stomatitis virus plaques on monolayers of human amnion (U) cells. One unit is the reciprocal of the dilution that reduces the plaque count by 50 per cent (24) All units are expressed in terms of the International reference preparation 69/19 (11) Confidence limits for the precision of the assay are around 95 per cent for 3 fold differences and exceed 99 per cent for 10 fold differences.

Chemical and Enzymic Treatments

The conditions for treatment of *HLI* were chosen by establishing the most vigorous that could be employed without destroying biological activity or sacrificing specificity of action. However in reaction 2 of Table 1 the combination of two such chemical treatments caused some destruction and the HLI injected into the rabbit contained only a third of the activity that it possessed before treatment.

Neuraminidase from *Clostridium perfringens* was purchased from the Sigma Chemical Company in the purest form available. It was allowed to react with HLI in 0 1M acetate buffer pH 5.0 at 37 C (1 unit liberates 1 μ mole N-Acetyl Neuraminic acid per min from *NAN* lactose or per 3 min from bovine sub-maxillary mucin, see Table 1 and figure legends for further details) A small amount of protease was present as a contaminant, but not enough to affect interferon activity under the conditions used. Reaction was terminated by lowering the pH to 2.0 with 2N *HCl*. After 15 min the pH was raised to 7 and the samples were stored at −20 C until assay when they were immediately diluted into the serum-containing assay medium. All HLI preparations are very stable at pH 2.0 (4)

Galactose Oxidase (E.C. No. 1 1 3.9) from *Dactylium dendroides* was purchased from the Sigma Chemical Company after partial purification. Reactions were carried out in 0.01M phosphate-buffered saline (pH 7.5) at 37 C, with 1 unit of enzyme to 5 million units of partially purified HLI, in the presence of catalase at 0.01 per cent to remove any hydrogen peroxide formed. Reaction was terminated after 30 min by the addition of diethyldithiocarbamate (at 2 mM) Neither catalase nor diethyldithiocarbamate had any effect of HLI under these conditions. Galactose oxidase was assayed using an o-tolidine/horse-radish peroxidase system (Worthington Biochemical Corporation 1 unit producing an adsorbance of one optical density unit at 425 nm using galactose as a substrate) Diethyldithiocarbamate was found to be fully effective as a selective inhibitor (1) and could be used to demonstrate that a contaminating enzyme activity in the galactose oxidase preparation was responsible for the destruction of interferon when more vigorous conditions of incubation were used.

Sodium meta periodate was used at a concentration of 5.5 mM at 4 C for 30 min in 0 1 M acetate buffer pH 4.0. The reaction was terminated by the addition of an 8 fold molar excess of ethylene glycol. Under these conditions side reactions should be minimal and its action confined to oxidation at vicinal hydroxyl groups of sugar residues (16) The antiviral activity of HLI is undisturbed by such treatment, but at higher temperatures and concentrations, and at longer incubation times, interferon is destroyed quite rapidly

Acid hydrolysis sialic acid is more easily hydrolysed from carbohydrates than other sugars are Heating at 80 C in 0 01N mineral acid can remove a substantial portion of sialic acid residues from various glycoproteins, e g (12) At pH 2.0 (0.05M glycine/*HCl* buffer) HLI can be heated at 75 C for 60 min without loss of activity (4) This is used as a chemical alternative to neuraminidase treatment, but no attempt was made to screen for hydrolysis products. Samples were dialysed against phosphate-buffered saline pH 7 5, before administration to rabbits.

Table 1 shows how these four reagents were used, singly and in combination, to treat *HLI* so that terminal sialic acid residues should be removed and exposed galactose residues destroyed.

Clearance of HLI from Rabbits

Test and control preparations of *HLI* prepared as described in Table 1 were injected into the marginal ear vein of rabbits, weighing 3.0 to 3.7 kg, in volumes not greater than 5 ml. Blood samples

TABLE 1 *Chemical and Enzymic Treatments Applied to Partially Purified Human Leukocyte Interferon (HLI) before its Administration to Rabbits*

Code	Treatment applied*	HLI millions of units injected
1	Acid hydrolysis	3.0
2	Acid hydrolysis + periodate	0.6
3	Periodate + acid hydrolysis	1.0
4	Periodate	1.5
5	Neuraminidase†	2.5
6	Neuraminidase† + galactose oxidase	3.0
7	Galactose oxidase + neuraminidase†	2.5
8	Galactose oxidase	4.5
9	Acid hydrolysis + galactose oxidase	2.5
10	Galactose oxidase + acid hydrolysis	1.0
11	Neuraminidase† + periodate	2.5
12	Periodate + neuraminidase†	2.5

* necessary changes of pH were accomplished by overnight dialysis against the appropriate buffer
† 1.0 unit of neuraminidase for one hour at 37 C, followed by one further unit and a further 30 min incubation.

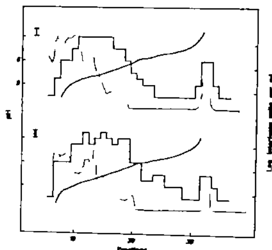


Fig 1 The effect of neuraminidase on the isoelectric profile of crude concentrated HLI I no treatment. II: treated with 0.01 units per million units of HLI for one hour at 37 C. Load per column 0.6 million units. Broken lines per cent transmittance, 1 cm cell, 280 nm. Solid line pH. Open blocks interferon expressed as log units/ml.

were removed, at times after for interferon assay (3) The clearance kinetics of the preparations described in Table 1 are shown in Fig 3.

Isoelectric Focusing

This was carried out on a LKB column (110 ml) with pH gradient 5–7 in 1 per cent ampholine, over a sucrose gradient 0–56 per cent (w/v) Samples were focused for 72 h at 7 C and potential of 450 V was applied. 3 ml fractions were collected, with automatic pH monitoring and diluted directly into medium for interferon assay

RESULTS

The isoelectric points of the main species of HLI are at acid pH, but considerable heterogeneity is evident (8) *Dorner et al.* (6) have suggested that variation in the sialic acid content of rabbit interferon molecules is responsible for their charge heterogeneity If a similar argument applies to HLI, then treatment with neuraminidase may be expected to alter the isoelectric profile, with a partial shift toward a higher pH and a distinct sharpening of the profile. Fig 1 shows the isoelectric profiles of crude concentrated HLI before and after neuraminidase treatment. The bulk of the material was obviously unaffected and the preparation appears at least as heterogeneous as before.

Fig 2 shows the effect on isoelectric profiles of treating partially purified HLI with two different concentrations of neuraminidase, as well as the effect of heating at 75 C for one h at pH 2.0 Again no major differences are obvious. The profile for acid hydrolysed HLI shows some evidence of shift of activity but the heterogeneity is, if anything, more pronounced. A peak focusing in the alkaline region and co-incident with a protein peak was present in all preparations of HLI regardless of purity or whether the interferon was treated or not. A similar peak is evident in the profile for a crude HLI previously reported by *Stanček et al.* (23) and would therefore appear to be a consistent feature of this type of interferon. Although different amounts of interferon were applied the recoveries in each case were very similar (between 50–100 per cent) It is clear from these data that sialic acid does not contribute substantially towards the heterogeneity of HLI.

Both chemical and enzymic treatments are used to either remove or destroy galactose and sialic acid, and the effects of such treatments on the antiviral activity, isoelectric points and pharmaco-kinetics of HLI are followed.

MATERIALS AND METHODS

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The conditions for treatment of HLI were chosen by establishing the most vigorous that could be employed without destroying biological activity or sacrificing specificity of action. However in reaction 2 of Table 1 the combination of two such chemical treatments caused some destruction and the HLI injected into the rabbit contained only a third of the activity that it possessed before treatment.

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Galactose Oxidase (E.C. No. 1.1.3.9.) from *Dactylium dendroides* was purchased from the Sigma Chemical Company after partial purification. Reactions were carried out in 0.01M phosphate-buffered saline (pH 7.3) at 37° C, with 1 unit of enzyme to 5 million units of partially purified HLI, in the presence of catalase at 0.01 per cent to remove any hydrogen peroxide formed. Reaction was terminated after 30 min by the addition of diethyldithiocarbamate (at 2 mM). Neither catalase nor diethyldithiocarbamate had any effect of HLI under these conditions. Galactose oxidase was assayed using an o-tolidine/horse-radish peroxidase system (Worthington Biochemical Corporation: 1 unit producing an adsorbance of one optical density unit at 425 nm using galactose as a substrate). Diethyldithiocarbamate was found to be fully effective as a selective inhibitor (1) and could be used to demonstrate that a contaminating enzyme activity in the galactose oxidase preparation was responsible for the destruction of interferon when more vigorous conditions of incubation were used.

Sodium meta periodate was used at a concentration of 3.3 mM at 4 C for 30 min in 0.1M acetate buffer pH 4.0. The reaction was terminated by the addition of an 8 fold molar excess of ethylene glycol. Under these conditions side reactions should be minimal and its action confined to oxidation at vicinal hydroxyl groups of sugar residues (16). The antiviral activity of HLI is undisturbed by such treatment, but at higher temperatures and concentrations, and at longer incubation times, interferon is destroyed quite rapidly.

Acid hydrolysis: sialic acid is more easily hydrolysed from carbohydrates than other sugars are. Heating at 80 C in 0.01N mineral acid can remove a substantial portion of sialic acid residues from various glycoproteins, e.g. (12). At pH 2.0 (0.05M glycine/HCl buffer) HLI can be heated at 75° C for 60 min without loss of activity (4). This was used as a chemical alternative to neuraminidase treatment, but no attempt was made to screen for hydrolysis products. Samples were dialysed against phosphate-buffered saline, pH 7.3, before administration to rabbits.

Table 1 shows how these four reagents were used, singly and in combination, to treat HLI so that terminal sialic acid residues should be removed and exposed galactose residues destroyed.

Clearance of HLI from Rabbits

Test and control preparations of HLI prepared as described in Table 1 were injected into the marginal ear vein of rabbits, weighing 3.0 to 3.7 kg, in volumes not greater than 3 ml. Blood samples

sist mainly of contaminant proteins (17) Thus the effects of chemical and enzymic treatments can only be followed by measuring biological activity and it is not possible to tell whether a change in the behaviour of HLI results from direct action on interferon, action on a contaminant which in turn affects activity or a mixture of both.

Our results cannot show that HLI is not a glycoprotein, nor can they demonstrate the complete absence of sialic acid and galactose residues. We cannot be certain that our treatments have succeeded in destroying all exposed galactose residues and it is still possible that residual amounts (26) are sufficient to sustain the characteristically rapid clearance kinetics.

On the other hand, we have not been able to obtain any evidence, whatsoever for the presence of carbohydrate in HLI None of the treatments described here had any effect on antiviral activity or upon its neutralization by specific antiserum (unpublished results) Less than 1 per cent of HLI will bind to the lectin, concanavalin A, and this proportion is not influenced by the treatments described in this paper The addition of concanavalin A or phytohaemagglutinin to treated or untreated HLI has no effect on either the expression of antiviral activity or its neutralization by antiserum (unpublished results)

It is possible that the administration of exogenous human interferon to a rabbit is not a suitable model for studying factors that influence its pharmaco-kinetics (3) HLI does, however exert antiviral activity in cultured rabbit cells (15) and possibly also in rabbits (19) The clearance of HLI in different species, including man is very similar and the levels of circulating HLI, at a given time, are proportional to the weight of the animal rather than degree of phylogenetic relatedness (5 22)

Rabbit interferon appears to contain terminal sialic acid and penultimate galactose residues (6) In HLI sialic acid does not appear to contribute to the molecular heterogeneity nor can we find evidence that galactose residues influence pharmaco-kinetics. The factors responsible for the heterogeneity of HLI are not known. Attempts to prolong the serum levels of administered interferon by the modification of putative carbohydrate residues would not seem appropriate for HLI

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HUMAN ECTHYMA CONTAGIOSUM (ORF) IN THE FAROE ISLANDS

An Epidemiological and Electron Microscopical Study

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"Hundaland" a disease occurring in the Faroe Islands, but of hitherto unknown aetiology was found to be very similar to ecthyma contagiosum (orf) An enquiry among general practitioners in the Faroe Islands confirmed this similarity and the disease was found to occur at an annual incidence of 25 per 40 000 inhabitants. Electron microscopy of biopsies from persons suffering from the disease revealed ecthyma contagiosum virus (orf virus) particles in all biopsies examined.

"Hundaland" is a disease occurring in the Faroe Islands, most frequently in the autumn. Although the connection between the disease and preceding contact with sheep has been known for a long time, its aetiology has hitherto been unknown.

The disease revealed many points of resemblance with ecthyma contagiosum, an infectious dermatitis of sheep and goats caused by the ecthyma contagiosum virus (orf virus) which belongs to the paravaccinia subgroup of the poxvirus group.

In the Faroe Islands, a skin disease of sheep, showing a clinical picture similar to that of ecthyma contagiosum, has been known for a long time as "skurv" (*Szabo* 1959) but it has not yet been examined whether "skurv" is identical with ecthyma contagiosum.

As a disease affecting human beings, ecthyma contagiosum seems to be known in most sheep-breeding parts of the world, but in the Scandinavian literature only one report is available in which three cases in Norway are described (*Hovding* 1957)

In order to examine the occurrence of "hundaland" in the Faroe Islands, an enquiry among the Faroese medical practitioners has been made. Further to examine whether "hundaland" is identical with ecthyma contagiosum, biopsies of lesions have been examined by electron microscopy in an attempt to find ecthyma contagiosum virus, and the following report contains the results obtained.

MATERIAL AND METHODS

The epidemiological study is based on an enquiry by letter among present and former general practitioners in the Faroe Islands in order to have all Faroese districts of medical practice represented. Seventeen questionnaires were sent out, 15 of which were answered.

Attempts to identify ecthyma contagiosum virus by electron microscopy were carried out on biopsies from 3 persons (case 1 2 and 3) suffering from a "hundaland" The diagnosis was made by general practitioners with many years of experience in medical practice in the Faroe Islands and based on the clinical picture of the lesion and the anam-

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CS

bably consisting mainly of DNA, was seen *to migrate into the spheres* which were almost filled by the dense material, leaving only an empty fringe along the shell (Figure 3) After *filling, the diameter of the shells measured* app 250 nm.

At the border of the virogenic matrix, clear and almost empty spaces *were generated in* the cytoplasm (Figure 2 & 4) At the border line, the developmental bodies were transformed into the mature form of the virion, now lying in the clear space. No distinct intermediate forms were observed.

If lying in the clear spaces, the mature virion would be in the shape of a flat, oblong ellipsoid, approximately *320* nm, *160* nm broad and 110 nm high, giving an axis ratio of about 2.0

The outline of the virion was profilated. Sections tangential to the surface of the virion revealed the profiling elements, which were *seen as approximately 10–12* nm broad, hollow threads or bands running parallel with an angle of patch of approximately 30–40 (Figure 3 & 6)

Separated from the profiled outermembrane by a faintly stained layer two membranes were surrounding the innerbody or core. Small lateral bodies inserted between these two *membranes—along the flat sides* of the virion—were observed, but normally *y* were not conspicuous.

The core was heavily stained. Seen from flattened side of the virion, the more faintly stained interior appeared to be divided by a wall or density sometimes in the shape of an oblongated S

The cytoplasm of the infected cells gradually changed into a clear space containing numerous mature virions, a rest of the matrix *and the emptied nucleus, leaving only a rim* of cytoplasm along the cell membrane (Figure 5) Still, the cells were able to go through an apparently normal process of keratinization. In the stratum corneum, the virions were embedded in a keratin matrix less dense than that in the periphery of the cell. Very flat virions as well as bended and twisted forms were more *common than in* the living cells. The virions were generally smaller being approximately 275 nm long, *140* nm broad and 80 nm high with an axis ratio about 1.95 (Figure 7)

In the material from the cases *1* and *3*, virus was found only in stratum corneum. In the material from case 3 virions were present *in a very high number (Figure 7) while* only a few virions were observed in the material from case 1 (Figure 8) In the material from case 2, virions were also very numerous but were lying in stratum granulosum and stratum spinosum (Figures 1–6)

It was only from the samples from case 2 that the survey sections could give some indication of the viral infection in that they showed empty-looking cells in stratum spinosum.

DISCUSSION

The Faroese name of the disease—"hundaland"—means mushroom, probably referring to the appearance of the lesion in the papillomatous stage where it may have some resemblance to a mushroom.

It is interesting to note that some persons have had "hundaland" twice at intervals of some years, thereby *indicating that* the disease is at least not followed by complete immunity

Fig 4 A few mature virions have appeared in the clear spaces *(CS) Note the filamentous inclusion* (arrow) in the nucleus (N) No nucleolus M: *matrix.*

Fig 5 Cell in stratum granulosum. Clear space (CS) containing a lot of mature virions has almost replaced the normal cytoplasm (C) a rest of which is left peripherally in the cell N nucleus.

Fig 6 Two virions partly tangentially sectioned. Note the profiling elements (arrows) of the outer layer Figure 1–6 from case 2

Fig 7 Mature virions, *from* case 3, embedded in keratine (stratum corneum) Note the serrated outline of the *profiled virion (arrow)* C core.

Fig 8. Virion *from* case 1 The round shadow (arrow) with a faint whorled pattern is possibly a tangentially sectioned end of a virion.


M
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5
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8

As appears from the above, there are many epidemiological and clinical points of resemblance between "hundaland" and ecthyma contagiosum. This applies to the previous contact with sheep the duration of the disease, its localization and appearance, the development of the lesion, and the complications.

Because ecthyma contagiosum (orf) virus is the only paravaccinia virus of ovine origin (*Nagington* 1968) and also because of the appearance and size of this virus, it seems relevant to compare the virus found with the said ecthyma contagiosum virus.

The characteristic feature of the virions is the outer coating, made up of threads arranged as if they were wound around the mature virions. The threads of the virus and their arrangement are by *Nagington et al.* (1964) described as components of the 25 nm thick outer membrane of the virion, their diameters being within the range of 8–10 nm. They describe the thread pattern as a bifilar lefthanded screw The same arrangement is found by *Büttner et al* (1964) who have measured the threads which were found to be 12 nm wide the pitch-angle being 45

The *profiling elements of the virus found* correspond very closely to the threads of orf virus described in the works mentioned above. Both *Buttner et al.* (1964) and *Peters et al.* [illegible]964) *state that the core of orf virus is* [illegible] longitudinally by two dense walls [illegible]orming a triplet of tubes or strands, while *Nagington et al.* (1964) suggest a coiled or roughly S-shaped configuration, but add that interpretation is not possible at present. The cores in our material are not of uniform appearance and we can find resemblance to all configurations mentioned, strongest, however to the type described by *Nagington et al.* (1964)

With regard to size and shape, the virus also corresponds to orf virus. *Büttner et al* (1964) have described the orf virus as 300 nm long and 170 nm broad, flattened cocoons with an axial *ratio of* 1 7± 20 *per* cent, while *Nagington & Horne* (1962) have measured it to be 263 nm long and 157 nm broad within the range of 220–300 nm. Our higher axial ratio may be explained by the circumstance that only normally shaped virions are measured and that virions selected for measurement on micrographs of sections are apt to be longer and narrower than those to be measured on micrographs of negatively stained specimens.

The observed morphological characteristics of the virus support the conclusion—based on the epidemiological data and the clinical picture—that the disease is ecthyma contagiosum.

This conclusion is also supported by the circumstance that sheep-breeding is by far the most important agricultural occupation in the Faroe Islands where the annual slaughterings amount to 40 000 30,000 of which are home-slaughterings in which many people participate. This could explain the high incidence (25 per 40 000) of this rather unnoticed disease, indicating again that ecthyma contagiosum may be very common among the sheep Therefore, if *practically* possible an investigation of "skurv" will also be carried out to determine its frequency and relation to ecthyma contagiosum in the Faroe Islands.

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ISOLATION OF PENICILLIN AND STREPTOMYCIN RESISTANT STRAINS OF *MORAXELLA OSLOENSIS*

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Two penicillin and streptomycin resistant *Moraxella* strains have been isolated from clinical specimens in Belgium. These strains were identified as *Moraxella osloensis* by comparison with known strains of this species in conventional, cultural and biochemical tests and in genetic transformation. Clinical details, bacteriological investigations, antibiotic sensitivity and genetic compatibilities with reference strains are reported. Penicillin sensitivity which so far has been used as an important criterion in the classification of *Moraxella* species, should not be taken to be an absolute requirement.

Moraxella osloensis has been identified as a separate species within the genus *Moraxella* (4 5 6 16) This species can be distinguished from other *Moraxella* species by different characteristics. One of the most striking features of *M osloensis* is its ability to accumulate poly-β-hydroxybutyrate as intracellular reserve material when cultured on mineral medium with DL-β-hydroxybutyrate as a sole source of carbon, which is not observed in other recognized *Moraxella* species (2 20) although such inclusions are found in the organism tentatively named *M urethralis* (18)

M osloensis as well as *M phenylpyruvica* were previously believed to belong to *M nonliquefaciens* (6 7) Since the taxonomy of the genus *Moraxella* has been studied and clarified only recently (2 6 7 16) few epidemiological data are available concerning each of the different species of the "*nonliquefaciens osloensis phenylpyruvica*" group Nevertheless, strains belonging to *M osloensis* have been isolated from many sources (2, 5 6, 19) but, with the exception of one report on the isolation of the organism from a case of septic arthritis (10) it has not been proved to be of pathogenic significance.

Two strains of penicillin and streptomycin resistant *Moraxella* spp., isolated from clinical material, have been examined. The first isolate appeared to be etiologically significant (9) while the second, clinically was of questionable significance.

As far as known, all hitherto studied *Moraxella* strains, including recently described new species (17 21 22) with the exception of three strains of *M phenylpyruvica* isolated in the United Kingdom (20) were found to be penicillin sensitive (2, 6 7 11 12, 13 14 15 19) *M osloensis* however has been found (2) to be slightly less sensitive to penicillin (M I C. less than 0.5 unit/ml) than the other species.

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The purpose of this paper is to record the isolation of the first penicillin and streptomycin resistant strains of *M. odoratus* and to examine by conventional, cultural and biochemical tests, and in genetic transformation whether these isolates are identical with typical strains of this species.

MATERIAL AND METHODS

Organisms

The two examined field strains were isolated in Brussels in 197 and have been designated as strain H102 and H161.

The *M. odoratus* strains A1920 (Type strain = NCTC 10465) and 5873 have been described previously by *Bøvre* (5).

Clinical Details

Case report No. 1 (Strain H102). A 2 ½-year-old boy was admitted to the hospital on account of severe stomatitis and impetiginous lesions scattered on his face, the back of the hands, and on the lips and gums; the tonsils were covered with a purulent exudate.

One week prior to admission, the patient had rhinitis, conjunctivitis, fever and cough; he was treated with 1 g tetracyclines orally daily.

Swabs from the throat, from the necrotic lesions of the lips and gums, and blood culture were obtained and sent to the laboratory for examination. Gram-stained films of the mouth lesions contained numerous leucocytes, mostly of the polynuclear type and an abundance of short, rod-shaped to coccoid Gram-negative organisms with a tendency to retain the crystal violet dye.

As superinfection was suspected after treatment with tetracyclines, the patient was treated with 2 g of ampicillin and g of oxacillin intravenously per day and also with 2.10⁶ Units of oral nystatin daily after the samples were taken. Neither penicillin nor streptomycin were administered to the child before the specimens for bacteriological examination had been taken.

Three days after admission, all these clinical samples yielded heavy and pure cultures of a *Moraxella* sp.

This clinical case has been reported in detail by *Butzler et al.* (9).

Case report No. 2 (Strain H161). The patient was a 1½-year-old girl, admitted to hospital on account of fever, meningeal signs, and enlargement of the cranial perimeter.

At the age of 6 months, she had a pneumococcal meningitidis, when she was 11 month old, a communicant hydrocephaly was diagnosed and a Pudenz' drain was inserted.

A lumbar puncture and a puncture at the level of the Pudenz' drain were performed. Examination of these clinical samples showed 1400 and 500 leucocytes, respectively, per cubic millimeter, mostly of the polynuclear type (95 per cent). Proteins amounted to 75 mg per cent in CSF and the glucose value ranged at 50 mg per cent. Gram-stained films and cultures were negative.

As a bacterial meningitidis was suspected, the patient was treated with Penicillin (4 × 150,000 Units daily) + Chloramphenicol (4 × 150 mg daily) + Gantrisin (4 × 500 mg daily).

Five days later no improvement was noticed and the treatment was changed to Methicillin (400 mg/kg daily) and Chloramphenicol (4 × 150 mg daily).

Eight days after admission, the disconnected Pudenz' drain was removed and sent to the laboratory for examination. A pure culture of a *Moraxella* sp. was obtained.

The girl became apyretic on the next day and on the 13th day of hospitalization antibiotic treatment was discontinued. A lumbar puncture performed on the 18th day showed a perfectly normal spinal fluid.

Morphological, Cultural and Biochemical Tests

The methods used by *Bøvre & Henriksen* (7) for the study of *M. phenylpyruvica* were utilized with slight modifications. Motility testing was performed by the hanging drop method of a 24 h old Heart Infusion Broth Culture. Characterization of colonies and detection of haemolysis were studied on 5 per cent horse blood agar. Kligler's Iron Agar was used instead of Triple Sugar Iron Agar. The strains were tested for growth on MacConkey agar, on Simmons's citrate medium, in nutrient broth containing 6.5 per cent and 2.5 per cent NaCl, and in Clark & Lubs' MRVP medium.

The strains were tested for ability to grow on the basal mineral medium of Owens and Keddie with 0.5 per cent (w/v) sodium acetate, and DL-β-hydroxybutyrate; a Sudan Black B staining served to detect whether the strains accumulate poly-β-hydroxybutyrate intracellularly. Tyrosinase activity and lecithinase production were studied on Trypticase Soy Agar (BBL) with 1 per cent (w/v) L-tyrosine and 10 per cent (v/v) egg yolk suspension.

Antibiotic Sensitivity Testing

The strains were tested for sensitivity to different antibiotics by the standard disc method of *Bauer et al.* (1). The minimum inhibitory concentrations (MICs) were determined by the classical dilution method in liquid media containing the following antibiotics: Penicillin G, Ampicillin and Tetracycline.

given before specimens were taken, and it cannot be excluded that a penicillin resistant mutant may have been selected But this explanation does not apply to the resistance to streptomycin since the latter was not used.

It was uncertain whether the streptomycin resistance of these two strains was of the single-step or the multi-step variety If it was of the latter type, one might have run into difficulties in transformation experiments. The fact that DNA from the two strains transformed the streptomycin sensitive strains in the same manner and to the same high resistance level, as DNA from a known single-step streptomycin resistant mutant of the type strain suggests that the two strains probably were single-step mutants.

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DIAGNOSIS OF RECENT *HERPES SIMPLEX* INFECTIONS

A Modified Immunofluorescent Test for the Detection of Specific Herpes Simplex *IgM Antibodies after Staphylococcal Adsorption of IgG*

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A modified, indirect immunofluorescent technique for the detection of specific serum *Herpes simplex* virus IgM antibodies is described. The previously necessary long incubation period with the serum for the determination of the IgM fluorescent antibody titre, is reduced from 3 to 1 hour after staphylococcal adsorption of IgG. This IgM test will also, in contrast to the previous procedure with untreated sera, give a more reliable and easy determination of the IgM titres, because the IgM fluorescence is more intense. Paired sera from 34 patients, taken approximately 6 and 15 days after the onset of symptoms, were examined. Sera from 11 patients showed a fourfold or greater rise in titre in the CFT and the IgG fluorescent antibody test, and 7 of these showed also a fourfold or greater rise in the IgM fluorescent antibody titre. The sera from the other patients, however, showed a constant titre in the CFT and the IgG fluorescent antibody test and were negative in the IgM test. The results show that in many cases a current *Herpes simplex* infection rapidly can be identified by this immunofluorescent technique.

Rapid confirmation of the diagnosis of a recent Herpesvirus infection, or particularly a herpetic encephalitis, has become increasingly important with the advent of a few rather toxic antiviral drugs such as iododeoxyuridine and cytosine arabinoside (5). Treatment with antiviral drugs is based on an early specific diagnosis. Usual diagnostic methods, such as virus isolation and determination of a significant rise in titre in the complement fixation test (CFT) are not suitable for rapid diagnosis. We have, therefore, developed a modified immunofluorescent antibody test for the detection of *Herpes simplex* virus, type I IgM antibodies, which may be detected during the first week after onset of symptoms.

In order to determine the maximal IgM fluorescent antibody titre, a long incubation period with the sera on the slides is necessary (10). High IgG concentrations may block the antigenic determinants of the virus before the IgM antibodies can react, partially because of the slower diffusion rate of the IgM molecules as compared to that of the IgG molecules. This effect may be either reduced by isolation of the IgM fraction or by a longer incubation time under which a partial exchange takes place between the IgM and the IgG molecules.

In this modified IgM fluorescent antibody test (FAT) system, 95 per cent of the IgG is adsorbed by treatment of the sera with *Staphylococcus aureus* Cowan type I (7)

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before testing the IgM antibodies. The rest of the IgG content, due to the subclass IgG_3 which does not react with the staphylococ cal cell wall protein A (6) does not seem to interfere in the IgM FAT

MATERIALS AND METHODS

Preparation of bacteria. Staphylococcus aureus Cowan type I NCTC 8530 (S aureus) was grown in a culture medium based on the *Woodin* (14) modification of the "CCY medium" described by *Gladstone & van Heyningen* (3) The cultures were rotated at 150 rev/min for 18 hours at 37 C (1) The bacterial cells were harvested, washed with phosphate buffered saline (PBS) and resuspended in PBS containing 3 per cent formaline (7) The suspension was left for 30 minutes at room temperature, washed three times with PBS and diluted to a 50 per cent (v/v) suspension with PBS containing 0.02 per cent sodiumazide and stored at 4 C. Immediately before use, the bacteria were washed and diluted as described.

Procedure for staphylococcal adsorption of IgG 0.1 ml serum was mixed with 0 4 ml of a 50 per cent suspension of bacterial cells and incubated at 37 C for 5 minutes. The mixture was then centrifuged and the concentrations of IgG and IgM in the supernatant were determined by single radial immunodiffusion in commercial Le-partigen plates (Behringwerke AG) containing specific antiserum against either human IgG or IgM.

Herpes simplex infected cells Confluent monolayers of the RK 13 cell line were grown in Roux bottles and infected with a high multiplicity of *Herpes simplex* type I (HS) The bottles were incubated at 37 C and the cells were removed after full degeneration, with 0.02 per cent versene washed three times with PBS and diluted to final concentration of 10^6 cells/ml. The HS infected cells were mixed with uninfected cells in the proportion of 1.20. If not used immediately the cells were suspended in foetal calf serum containing 0.5 per cent dimethylsulphoxide and stored at −70°C for up to six months. Before use the cells were washed twice with PBS

Preparation of slides The slides were prepared as described by *Rafkai et al.* (8) and each well on the glass surface was filled with 0.1 to 0.2 ml of the suspension of HS infected cells. The slides were air dried and fixed in CCl for 10 minutes at 4 C. The CCl was evaporated and the slides were stored at −70 C if not used immediately

Titration of sera The sera were titrated in twofold dilutions in the IgG and the IgM FAT starting at 1 10 and 1.5 respectively As the HS IgG titre was easier to determine in fourfold, rather than in a twofold dilution series, the IgG FAT was divided into different fourfold dilutions on two slides. However when the first dilution on the two slides was 1 10 and 1:20 respectively the IgG titre was, like the IgM titre, read as a twofold dilution series.

Staining procedure The cell smears were overlaid with 0.1 ml of the serum dilutions and incubated for one hour in a moist chamber at 37°C, and then washed with four changes of PBS for a total of one hour However when using untreated sera, the incubation period for determination of the IgM titre was three hours. The cell smears were then covered with 0.1 ml of the working dilutions of either sheep antihuman IgG or sheep antihuman IgM conjugates and incubated and washed as previously described. The working dilutions of the conjugates, labelled with fluorescein isothiocyanate (Wellcome Reagents Ltd.) were 1.50 and 1 20 respectively Finally the slides were mounted in buffered glycerol at pH 9.0. In each test a positive and a negative control serum were included.

Reading the fluorescent antibody test The prepared slides were examined on a Leitz, Ortholux, microscope, and only a typical yellowgreen cytoplasmic fluorescence was taken into account. The serum antibody titre is defined as the reciprocal of the highest dilution, giving definite fluorescence in the cytoplasma of the infected cells.

Ultracentrifugation of serum. For separation of the IgM antibodies from the other serum immunoglobulins, 1 ml serum was layered on a sucrose gradient, 10 to 40 per cent (w/v) in PBS, and centrifuged at 10^4 × g for 28 hours at 4°C. Twenty-two fractions of 1.3 ml were collected.

The CFT was carried out according to the method described by *Takatsy* (13) and *Sever* (11) in twofold dilutions from 1.5

RESULTS

The optimal test conditions were determined in some preliminary experiments.

Staphylococcal adsorption of serum IgG The adsorption capacity of IgG by *S aureus* was tested and a maximal adsorption of 95 per cent of the serum IgG was obtained by use of a 50 per cent suspension of bacterial cells. Studies of the specific HS IgG fluorescent antibodies in the adsorbed sera, showed a similar reduction in the HS IgG antibody content.

For the adsorption of IgG *Lind & Mansa* (7) used an incubation time of 45 minutes at 37 C. In this test system, the adsorption

TABLE 1 *The IgM Fluorescent Antibody Titres in Adsorbed and Untreated Sera after Different Incubation Periods on the Slides*

Serum		IgM FAT titres			
		Untreated serum		Adsorbed serum	
Patient	No.	1 hour	3 hours	1 hour	3 hours
1	I	<5	5	10	10
	II	10	20	40	40
	I	10	10	10	10
	II	20	40	160	160
3	I	10	20	40	40
	II	40	160	320	320
4	I	5	10	10	10
	II	20	40	40	40
5	I	5	20	40	40
	II	10	40	160	160
6	I	5	10	10	10
	II	40	80	320	320
7	I	10	20	40	40
	II	10	40	160	160

I, the acute phase serum.
II, the convalescent phase serum.

of IgG was estimated after an incubation time of 5–45 minutes at 37 C. Within this period, no detectable difference in the adsorption of IgG was found. As determined by radial immunodiffusion, the content of IgM was reduced 10–15 per cent after staphylococcal adsorption of IgG

The fluorescent antibody test system To check the specificity of the conjugates, the different immunoglobulins in a patient serum were separated by ultracentrifugation in a sucrose gradient. The fractions, collected from the gradient, were analyzed for the contents of IgG and IgM by radial immunodiffusion and of specific HS IgG and IgM fluorescent antibodies No detectable cross reactions between the labelled anti-IgG and anti-IgM were seen, and regarding the conjugates, this indicated a high degree of specificity

To determine the optimal serum IgM titre a positive IgM serum, untreated and adsorbed with *S aureus* was incubated on the slides from ½–4 hours. Every half hour one slide from the untreated and the adsorbed serum was stained, and the maximal titres were reached after 3 and 1 hour respectively In addition, the IgM antibody titre level was generally higher by use of the adsorbed sera (Table 1)

The specificity of the HS FAT was checked with paired sera showing a significant rise in titre in the CFT against *Cytomegalovirus Varicella zoster* and *Adenovirus* and with sera with a fourfold or greater rise in the fluorescent antibody titre against *Epstein Barr* virus capsid antigen (4) However no cross reacting antibodies were found. Some sera with a rise in the CFT titre against both *Varicella-zoster* and HS also showed a rise in the HS IgG fluorescent antibody titre, and similar results have been shown by *Schmidt et al* (9)

Patient analysis Paired sera from 34 patients were examined for HS complement fixing antibodies, and HS IgG and IgM fluorescent antibodies. The IgM titre was determined in adsorbed sera.

TABLE 2. *Serological Data for Paired Sera from Patients with a Recent HS Infection*

Patient Sex	Patient Age	Symptoms, diagnosis	Days after onset of symptoms	Antibody titre CFT	Antibody titre IgG FAT	Antibody titre IgM FAT
F	25	Herpes genitalis	7	10	80	10
			27	40	320	40
M	18	Stomatitis angina	7	20	80	10
			25	160	1280	160
F	1	Encephalitis	15	40	320	40
			22	≥ 320	5120	320
F	69	Herpes facialis febris	8	<5	320	10
			15	40	1280	40
F	18	Encephalitis	6	10	1280	40
			15	40	5120	160
F	8	Herpangina	7	<5	40	10
			15	80	5120	320
F	1	Stomatitis	5	20	320	40
			12	≥ 320	5120	160
M	23	Meningitis serosa	–	<5	160	<5
			–	10	1280	<5
F	7	Herpes labialis	5	20	640	<5
			9	80	5120	<5
F	28	Encephalitis	11	20	640	<5
			30	80	10240	<5
F	25	Meningitis serosa	15	20	320	<5
			25	≥ 320	1280	<5

TABLE 3 *Summary of the HS Antibody Titres in Paired Sera from 11 Patients with Recent Infection and from [illegible] Patients with an Earlier Infection*

gnosis	Serum No.	Serum Days after onset of symptoms	Antibody titre CFT	Antibody titre IgG FAT	Antibody titre IgM FAT
Recent infection	I	5–10	<5– 40	40– 1280	<5, 5– 40
	II	10–30	40–≥ 320	320–10240	<5, 40–320
Earlier infection	–	–	10 160	640– 2560	<5

Eleven paired sera with a significant rise in titre in the CFT also showed a fourfold or greater rise in the IgG fluorescent antibody titre In addition 7 of these paired sera showed specific HS IgM antibodies in the first serum and a significant rise in the IgM titre The results and information about age, sex and clinical diagnosis of the patients are giving in Table 2. The other paired sera from 4 children and 19 adults, with a constant titre in the CFT and the IgG FAT were negative in the HS IgM FAT

According to the generally accepted criteria for a recent, or an earlier viral infection, the results may be summarized in two groups. The recent infection with a fourfold or greater rise in titre and the earlier infection with a constant titre in the CFT as shown in Table 3

Fourteen sera, positive in the Hyland Latex test for rheumatoid factor were tested in this HS FAT and 10 of these showed a positive IgM fluorescence However after adsorption of IgG the sera were negative in the IgM test.

DISCUSSION

The modified HS IgM FAT will, in contrast to the previous procedure with untreated sera, give a more reliable and easy determination of the titre, because both the IgM titre level and the intensity of the fluorescence are greater This is important, when testing the acute serum only where a positive IgM titre is at a low level.

It has been shown that the rheumatoid factor in some cases can give positive IgM fluorescence in cells infected with different viruses (2, 12) This type of reaction should be detected by examining the IgM titre in both the untreated and the adsorbed sera. If the rheumatoid factor is responsible for the IgM fluorescence, the titre will be lower in the adsorbed sera than in the untreated ones. Such a decrease in the IgM titre was not detected in these sera with a significant rise in the CFT and a possible nonspecific fluorescence due to the rheumatoid factor could therefore be excluded.

The results also show that the specificity of the HS IgG FAT is much the same as the specificity of the CFT However the sensitivity of the IgG FAT is much higher

The outlined indirect HS FAT used in these analyses, has a high degree of specificity and no cross reactions with other viral antibodies were found. It should be mentioned however that sera with a rise in the CFT titre against both *Varicella zoster* and HS also showed a rise in the HS IgG FAT titre (9) This indicates a similar specificity of the HS IgG FAT and the CFT as previously mentioned.

The results of the HS CFT and FAT combined with clinical observations will, in many instances, give an early diagnosis. A positive IgM FAT in the serum will probably indicate a current infection, because no detectable HS IgM antibodies were found in the other paired sera with constant CFT and IgG FAT titres. Also a moderate CFT titre and a IgG FAT titre lower than 640 in the acute serum will possibly indicate a recent infection even if IgM antibodies are not detected. In contrast, the IgG FAT titres among 22 patients with a previous infection are stabilized at a titre between 640 and 2560.

According to these results, a current HS infection may be diagnosed within the first week after onset of symptoms, and treatment can be started in the acute phase of the illness.

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THE ULTRASTRUCTURE OF CULTIVABLE TREPONEMES

I Treponema phagedenis, Treponema vincentii *and* Treponema refringens

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Treponema phagedenis, *Treponema vincentii* and *Treponema refringens* were studied in the electron microscope by means of negative staining and sectioning techniques. The length and width of the cells were within the same limits for all three strains examined. Statistical analysis, however, demonstrated a difference in the wavelengths of the cells. Organisms of *T. phagedenis* and *T. vincentii* had blunt ends and possessed cell wall surface layers in which a regular substructure was only occasionally revealed, while cells of *T. refringens* had tapered ends and a distinctly structured cell wall surface layer. Generally cells of the strains investigated had 4–6 flagella inserted at each end. Two bundles of flagella, one from each end of the cell, wound around the organism and overlapped in the middle of the cell. The flagella with their insertion organelles were identical for the three strains studied. Two bundles of cytoplasmic tubules were detected in the interior of the treponemes after treatment with either sodium deoxycholate or *Myxobacter* AL-1 protease I. The two bundles of cytoplasmic tubules overlapped in the middle of the cell. The results of the present morphological study are compared with results obtained by other investigators studying other characteristics of these strains and it is concluded that the three strains studied belong to three different species.

Previous studies employing electron microscopy have demonstrated some morphological differences between pathogenic, non-cultivable treponemes and non-pathogenic, cultivable treponemes (8, 9, 13, 15, 19). It appears from the literature that morphological differences also seem to exist between different strains of cultivable treponemes (2, 3, 5, 12, 14, 15). However, up to now investigators have examined different strains of the species, and various methods of preparation for electron microscopy have been applied, which make a direct comparison of results somewhat difficult.

For some years a comparative study of the ultrastructure of several species of the genus *Treponema*—the majority of which were originally isolated from the genitalia of human beings—has been carried out in this laboratory. During this period identical methods for negative staining and sectioning for electron microscopy were used throughout in order to compare the ultrastructure of the species studied. We hoped that these standardized procedures of the modern electron microscopy laboratory would disclose differences and/or reveal similarities which could be of potential use for the classification of individual members of the genus.

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The results of ultrastructural studies on *T phagedenis*, *T vincentii* and *T refringens* are presented in this paper

MATERIALS AND METHODS

The strains of *T phagedenis* and *T refringens* used for this investigation were kindly supplied by Dr *P Thibault*, La Collection de l'Institut Pasteur, Paris, France. The strains were labelled CIP 5163 and CIP 5164 respectively and were isolated from genital lesions by Dr *R. Vincent* in 1934 (12) *T vincentii* labelled *Borrelia vincentii* N9 was obtained from Professor *P H Hardy*, Department of Microbiology, Johns Hopkins University, Baltimore, USA. Originally Professor *Hardy* obtained the strain from Professor *E G Hampp*

The treponemes were grown at 36 C in an atmosphere of 95 per cent N_2 and 5 per cent CO_2 in a thioglycollate medium of a composition described earlier (7) The cells were harvested after 1 week by adding one volume of a sucrose solution containing magnesium and calcium ions (SMC)* to one volume of the culture and subsequent centrifugation for 15 minutes at about 7000 × g. The pellets thus obtained were resuspended in SMC to a suitable density and immediately treated as stated below

In some preparations organisms were fixed by adding one volume SMC + 1/10 volume 3 per cent glutaraldehyde in SMC to one volume of the culture. Fixation time was 25 minutes including centrifugation as stated above.

Preparation for Negative Staining

Samples of cell suspensions were treated on specimen grids for electron microscopy with 0.2 per cent Teepol or 1 per cent sodium deoxycholate both in redistilled water or with a solution of *Myxobacter* AL-1 protease I** (AL-1 enzyme) containing 100 μg/ml of the enzyme and 5 × 10^{-} M EDTA in 2.5 × 10^{-2} M TRIS buffer pH 9.0. All preparations were stained with 1 per cent (w/v) ammonium molybdate adjusted to pH 7 with NH_4OH. The procedure used for treatment and negative staining was a multiple drop technique described previously (7) and the times for treatment with the active agents varied from 30 seconds to 4 minutes.

Flagella were liberated from the treponemes and concentrated by the following procedure. One volume of SMC was added to one volume of culture and the mixture was centrifuged at 7000 × g for 15 minutes. The pellet obtained was resuspended in two volumes of redistilled water and again centrifuged for 15 minutes at 7000 × g. The pellet was then suspended in two volumes of 0.2 per cent Teepol in redistilled water and left at room temperature for 20 minutes before the suspension was centrifuged at 7000 × g for 20 minutes. The pellet from this centrifugation consisted mainly of cytoplasmic bodies of the cells and was discarded. The free flagella present in the supernatant were concentrated by centrifugation at about 40,000 × g for 1 hour and the pellet was resuspended in a few drops of SMC. Samples of this suspension of flagella were negatively stained for electron microscopy as stated above.

Preparation for Sectioning

10 ml SMC + 1 ml 3 per cent glutaraldehyde in redistilled water were added to 10 ml of a 1 week old culture and prefixation was carried out for 30 minutes, inclusive centrifugation at about 11,000 × g for 10 minutes. The pellet obtained was enrobed in 3–4 drops of warm, melted agar at 45 C (1.5 per cent Noble Agar Difco in SMC). Agar blocks of about 1 mm were cut from the solidified agar and post fixed overnight at room temperature in 1 per cent OsO4 in SMC, to which was added 10 per cent YAP medium (yeast extract-sodium acetate-peptone medium 0.5, 0.05 and 0.5 per cent, respectively of the Difco products). After a brief wash in SMC the blocks were treated for 1 hour at room temperature with 2 per cent uranyl acetate in SMC (17) then dehydrated in alcohol and propylene oxide (11) and embedded in Vestopal-W (18)

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*The SMC solution consisted of 0.03 per cent sucrose, 0.01 M $MgCl_2$ and 0.01 M $CaCl_2$ in redistilled water. The pH was not adjusted but was usually about 5

**The purified bacteriolytic enzyme of *Myxobacter* AL-1 was kindly provided by Professor *R S Wolfe*, Department of Microbiology, University of Illinois, Urbana, Ill., USA.

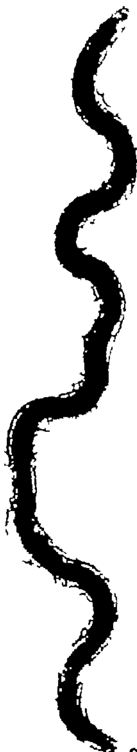
Figs 1–13, 16–19 and 22–28 all show negatively stained material. Figs 14–15, 20–21 and 29–30 all show sectioned material. The bar on each micrograph represents 100 nm.

Fig 1 *T phagedenis*. Unfixed organism with regular waves. The treponeme is covered with an amorphous outer layer (arrows). Note the blunt ends of the treponeme. × 30 000

Fig 2 *T vincentii*. Unfixed organism with somewhat irregular waves. The treponeme has blunt ends. × 30,000

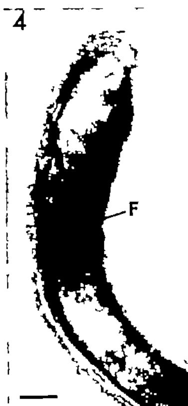
Fig 3 *T refringens*. Unfixed organism with regular waves. Note the tapered ends of the treponeme. × 30,000.







1 2


4
F

5
F

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F

7
T

8
T

9
T
F

Sectioning and Electron Microscopy

Ultrathin sections were obtained on the LKB ultrotome I or III microtomes and were collected on formvar coated carbon reinforced copper grids. Sections were stained for 15 minutes with magnesium uranyl acetate (4) and for 2 minutes with lead citrate (16)

Grids with sectioned as well as negatively stained material were examined in Philips EM 200 or EM 300, electron microscopes. Micrographs were taken on Kodak Fine Grain Release Positive Film Type 5302 at initial magnifications of 9000 × and 16000 × and were enlarged photographically as desired.

For this paper pproximately 800 recordings were studied.

RESULTS

Treponema phagedenis

Cells of *T phagedenis* were found to be rather regularly coiled (Fig. 1) Their length, measured along the axis of the helices, varied between 6–12 μm for single cells, and the width from 0.20–0.25 μm whether measured on negatively stained or on sectioned cells. The mean wavelength was measured to 1 6 μm and the amplitude to 0.2–0.3 μm. All cells were covered with an amorphous outer layer (Fig 1) The cells had blunt ends with flagella inserted terminally and haphazardly (Fig 4) The number of flagella inserted at each end of an organism was usually 4–6 (Fig 4) but cells with as few as 3 or as many as 7 flagella inserted at each end have been observed. Two bundles of flagella, one from each end, were twined together with the cytoplasmic body of the treponeme. The bundles overlapped in the middle of the cell and individual flagella of each bundle interdigitated in this region.

Treatment of *T phagedenis* cells with 1 per cent sodium deoxycholate for 30 seconds revealed bundles of thin fibrils in the interior of the cells (Fig 7) A bundle consisted of 6–8 fibrils (Figs. 7 10, 13) each with a diameter of about 7 nm and a bundle extended from each end of individual cells. The bundles of these thin fibrils were found to be wound in the interior of the treponemes in a manner similar to that of flagella around the cytoplasmic body of the cells. The bundles were so long that the individual fibrils of each bundle showed interdigitation in the middle region of the cells. In cells treated with AL-1 enzyme, these bundles of thin fibrils were more easily observed. On close inspection of micrographs obtained at a high magnification, heavy metal salt was seen to have penetrated into the central part of individual fibrils thus demonstrating their tubular nature (Fig. 13) In addition these fibrils or cytoplasmic tubules must be rather fragile as they often appeared broken*

Fig 4 *T phagedenis*. The flagella (F) are inserted terminally and haphazardly Note the blunt end of the treponeme From a glutaraldehyde fixed preparation. × 90,000.

Fig 5 *T vincentii*. An indication of a substructure in the outermost layer of the organism is seen (arrow) The flagella (F) are inserted terminally and haphazardly From an unfixed preparation. × 90,000.

Fig. 6 *T refringens*. A regularly structured surface layer (arrow) covers the cell. Flagella (F) are inserted subterminally and haphazardly From an unfixed preparation. × 90,000.

Figs 7–9 show cytoplasmic tubules revealed in treponemes treated with sodium deoxycholate for 1 minute (Fig. 8) or 2 minutes (Figs 7 & 9)

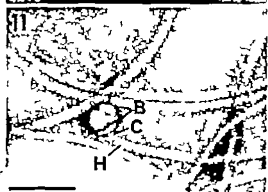
Fig. 7 *T phagedenis*. Cytoplasmic tubules (T) are easily depicted. Some flagella (F) are also present. × 90,000.

Fig. 8 *T vincentii*. Cytoplasmic tubules (T) are seen close to the insertion point of a flagellum (arrow) × 90,000.

Fig. 9 *T refringens*. In addition to cytoplasmic tubules (T) and flagella (F) some thin fibrils (arrows) are seen in association with remnants of the outer layer of the treponeme × 90,000.

* At the meeting of the Subcommittee on Taxonomy of Spirochaetales of I.S.C.B. at the First International Congress for Bacteriology Israel 1973 it was generally agreed to use the term cytoplasmic tubules for the intracytoplasmic 7 nm thick tubular structures observed in cells of several species of this group. This was agreed to in order to avoid confusion with the 15–30 nm microtubules ubiquitously present in animal and plant cells of higher orders. Consequently the cytoplasmic tubules described in the present paper are structures identical to those called microtubules in previous papers of this series.

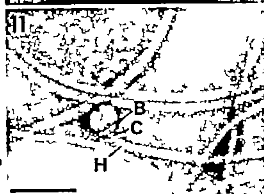




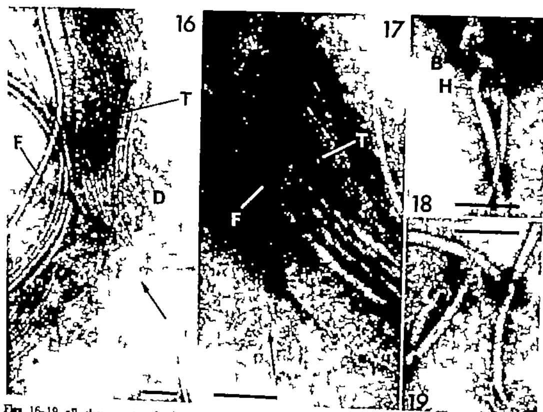


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F
D
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T


10
T
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Z
D


11
B
C
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12
B
H
C



Figs 16–19 all show material obtained from *T vincentii*.

Fig 16. Part of a treponeme treated with *Myxobacter* AL-1 protease 1 for 30 seconds. Only flagella (F) cytoplasmic tubules (T) and membranous debris (D) are left. In the lower middle part of the field the cytoplasmic tubules appear to be broken. A regular substructure can be seen on some of the membranous debris (arrow) × 90,000.

Fig 17 Part of a treponeme treated as for Fig. 16 but for 4 minutes. A bundle of flagella (F) and a bundle of cytoplasmic tubules (T) are seen. Pieces of the cell wall surface layer show a regular substructure (arrow) × 160,000.

Fig 18. A sheathed and an unsheathed flagellum liberated after treatment of a suspension of treponemes with Teepol. The basal knob (B) the collar (C) and the hook (H) are present. × 160,000.

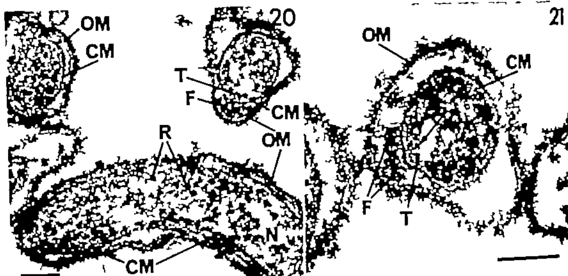
Fig 19 Flagella liberated from cells treated as for Fig. 17 Ring-shaped structures surround the basal ends of the flagella. × 160,000

where individual flagella from either end of the cell interdigitated.

Bundles of cytoplasmic tubules were revealed in cells treated with either sodium deoxycholate (Fig 8) or AL-1 enzyme (Figs. 16 & 17) The cytoplasmic tubules were often found to be broken (Fig. 16) In preparations of cells treated with the detergents or with AL-1 enzyme cellular debris which probably originated from the cell wall surface layer was found quite frequently The debris showed a substructure similar to that observed on the regularly structured cell wall surface layer of *T refringens* (see later) (Figs. 16 & 17)

Flagella isolated from *T vincentii* cells were in all respects similar to those isolated from cells of *T phagedenis* (Table 1 Figs. 18 & 19

Sectioned cells of *T vincentii* were found to be surrounded by an ordinary triple-layered outer membrane to which a rather thick electron dense layer was attached to the exterior side (Figs. 20 & 21) The cytoplasmic



Figs. 20–21 T. vincentii. The parts of sectioned treponemes illustrated show that the flagella (F) are situated between the asymmetrical outer membrane (OM) and the asymmetrical cytoplasmic membrane (CM). Ribosomes (R), a nuclear region (N) and cytoplasmic tubules (T) are present in the cytoplasm of the organisms. The cytoplasmic tubules appear to present less electron dense centres (arrow Fig. 21). Fig. 20 × 90,000. Fig. 21 × 175,000.

body was bordered by an asymmetrical triple layered cytoplasmic membrane in which the outer dark layer was somewhat wider than the inner (Figs. 20 & 21). The flagella were situated between the cell wall and the cytoplasmic membrane (Figs. 20 & 21). The cytoplasmic tubules were in a few sectioned cells detected in the cytoplasm close to the inner leaflet of the cytoplasmic membrane and always exactly underneath the flagella (Figs. 20 & 21). On a few micrographs the cytoplasmic tubules presented less electron dense centres (Fig. 21). Ribosomes and nuclear material could be identified in the cytoplasm of the cells (Fig. 20).

Treponema refringens

Cells of *T. refringens* were found to be regularly coiled from 4–12 μm long and approximately 0.23 μm wide (Fig. 3). The mean wavelength was 1.8 μm, the amplitude was 0.2–0.3 μm, i.e. the same as for *T. phagedenis* and *T. vincentii*. The cells were covered by a regularly structured outer layer (Fig. 6). The ends of the cells were slightly tapered (Figs. 3 & 6) with 4–5 flagella inserted haphazardly and subterminally at each end (Fig. 6). The outermost flagellum was generally inserted 0.15 μm from the end of the cytoplasmic body of the organism. Also for this treponeme the flagella were arranged in two bundles which wound around the cytoplasmic body of the cell. The two bundles overlapped in the middle of the cell where

Figs. 22–24 all show material obtained from *T. refringens*.

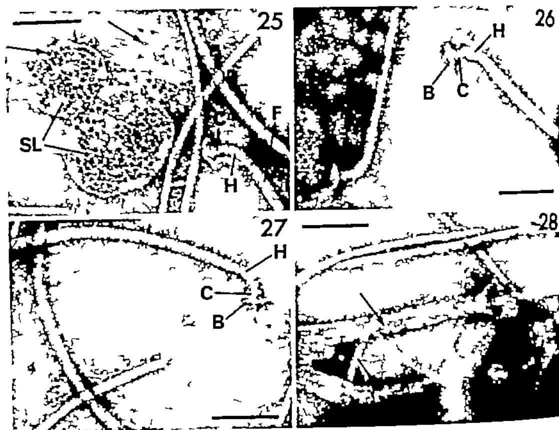
Fig. 22. Part of a treponeme treated with *Myxobacter* AL-1 protease 1 for 2 minutes. The two bundles of flexible cytoplasmic tubules overlap (arrow). The regularly structured surface layer covers the remnants of the cell. F denotes sheathed flagella and U some unsheathed. × 90,000.

Fig. 23. Remnants of treponemes treated in suspension with Teepol. The substructure of the surface layer (SL) consists of hexagonally packed rings. In the centre of some of the rings points of less electron density are visible (arrows). Note the honeycombed substructure on the hook (H) of the flagellum (F). × 160,000.

Fig. 24. Ring-shaped structures of the surface layer (SL) in close association with thin fibrils. From the same organism as on Fig. 9. × 175,000.


23
SL
F
H


SL



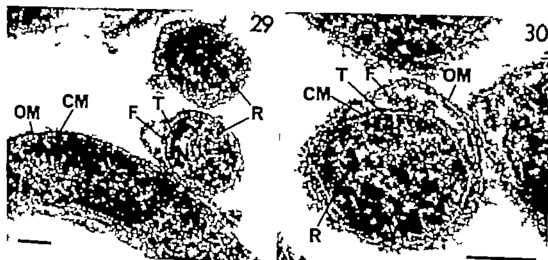
Figs 25–28 all show preparations of *T refringens* treated as for Fig. 23

Fig 25 The substructure of the hook (H) differs both from that of the collar (C) and that of the shaft (F) The hexagonal pattern of the rings is seen on pieces of the surface layer (SL) Less electron dense points are present in the centres of some of these rings (arrows) × 160,000

Figs 26–27 The basal knobs (B) seem to consist of two narrow discs in very close association. The collars (C) and the hooks (H) are also illustrated. × 160,000

Fig 28 Parts of two flagella more or less surrounded by striated tubules (arrows) (see text) × 160,000

individual flagella of each bundle interdigitated. Treatment of cells of *T refringens* with 1 per cent sodium deoxycholate or AL-1 enzyme revealed two bundles of cytoplasmic tubules within the cells (Figs. 9 & 22) Very few cytoplasmic tubules appeared to be broken (Fig 22) In addition to cytoplasmic tubules and flagella, some thin fibrils with a diameter of about 4 nm were associated with cells treated with sodium deoxycholate (Fig 9) The regular surface structures seen on untreated cells were also found in preparations of cells treated with AL-1 enzyme (Fig 22) and with Teepol (Fig 23) The regular pattern seemed to be produced by small rings aligned in tightly packed rows. The rings in one row appeared to be displaced at a distance of one radius sidewise with respect to the rings on the two adjacent rows. In this way a sheet of hexagonally packed ring-shaped structures seemed to constitute the regular surface layer (Fig 23) A point of lesser electron density was seen in the centre of most of the rings (Fig 23) Some micrographs showed an indication of the rings being connected to or in close association with some thin fibrils which were situated between the rows of the rings and running parallel to them (Fig 24) The diameter of these fibrils was about 4 nm, and the centre-to-



Figs. 29–30 T. refringens. The parts of sectioned treponemes illustrated show that the exterior layer of the outer membrane (OM) is more electron dense than the inner. The cytoplasmic membrane (CM) is also asymmetric. Flagella (F) are situated in the interspace between the outer membrane and the cytoplasmic membrane. Cytoplasmic tubules (T) are seen in close apposition to the cytoplasmic membrane. Ribosomes (R) and a nuclear region (N) are also present. Fig. 29 × 90,000. Fig. 30 × 175,000.

centre distance between them was 10 nm, which is the same as that between the centres of the rings.

The organelles observed on isolated flagella were similar to those observed on flagella isolated from cells of *T. phagedenis* and *T. vincentii* (Table 1, Figs. 25, 26, 27) and the substructures of these as well as their dimensions were also identical. The flagella and their sheaths also had the same dimensions as the flagella isolated from the two other treponemes studied. These results were similar irrespective of whether the flagella were obtained from cells treated with AL-1 enzyme or from cells treated with Teepol. There was an indication that the basal knobs consisted of two narrow rings or plates situated a few nm apart (Figs. 26 & 27). Some isolated flagella were found to be surrounded by striated tubules (Fig. 28) of the same type as those previously described for some other spirochetes (1, 6). In the present study the periodicity of the striations on these tubules was found to be about 5 nm.

Sectioned cells of *T. refringens* appeared to be very similar to those of *T. phagedenis*. They were surrounded by an asymmetrical three-layered membrane with the outer electron dense layer slightly wider and more electron dense than the inner (Figs. 29 & 30). The cytoplasmic membrane also appeared to be asymmetric, with the outer electron dense layer somewhat wider than the inner (Figs. 29 & 30). The flagella were situated in the space between the cell wall and the cytoplasmic membrane. Ribosomes and nuclear regions could be seen in the cytoplasm of the cells. Cytoplasmic tubules were identified in the cytoplasm of some cells and were situated in a manner identical to the cytoplasmic tubules of cells of *T. phagedenis* and *T. vincentii* (Figs. 29 & 30).

DISCUSSION

The dimensions of the various morphological details were compiled (Table 1) in order to analyse any possible differences between cells of the species studied. From this table it is evident that only the wavelength of the cells of one species differs from the values obtained from cells of the two other species. The wavelengths were calculated and the results analysed as follows. The wavelength of each cell was calculated as the cell length divided by the number of waves per cell.

TABLE 2. *Mean Wavelengths and Variances for* T phagedenis T vincentii *and* T refringens

Strain	Mean ($\bar{x}$) wavelength	Variance	Number of cells measured
T phagedenis	1.63	0.0253	6
T vincentii	1.41	0.0531	10
T refringens	1.83	0.0227	16

These values were plotted against the cell length and for all three strains it was found that the wavelength was independent of the cell length (the diagram is not shown)

Assuming that the wavelength for cells belonging to the same strain shows a normal distribution around a mean value it can be tested by a one-way analysis of variance whether the three strains have the same mean wavelength. The variances are assumed to be identical for the three strains, although for the cells of *T vincetii* as can be seen from Table 2 it is about twice as large as for cells of the two other strains. The hypothesis of a common mean wavelength ($\bar{x}$) therefore is to be rejected because, as shown in Tables 2 & 3

$$\bar{x}_{T\ vincentii} \leqq \bar{x}_{T\ phagedenis} < \bar{x}_{T\ refringens}$$

Without making any assumption about the type of distribution it can now be tested by the Wilcoxon test whether the strain *T vincentii* compared with *T phagedenis* and *T phagedenis* compared with *T refringens* have the same wavelength distribution. The results of this analysis show that cells of all three strains have different wavelengths (test level $\leqq$ 5 per cent) (Table 3)

The means, variances, and test levels are shown in Table 2 & 3 It is noteworthy that a statistical analysis is able to disclose differences in wavelengths for the negatively stained treponemes studied. In addition to this difference in wavelength a few characteristic morphological differences have been disclosed which make it possible to distinguish cells of *T refringens* from cells of *T phagedenis* and *T vincentii*.

1 Cells of *T refringens* have tapered ends with the flagella inserted somewhat subterminally whereas those of *T phagedenis* and *T vincentii* have blunt ends with more terminally inserted flagella.

2. The cell wall surface layer on cells of *T refringens* has a distinct regular substructure, whilst the cell wall surface layers of undamaged cells of *T phagedenis* and *T vincentii* usually appear to be amorphous.

Other differences between cells of *T refringens* and those of *T phagedenis* and *T vincentii* are

a. The attachment of flagella to cells of *T refringens* is more resistant to Teepol treatment than is the case for flagella on cells of the two other species. Only few flagella are released from the cells after treatment on grids for 30 seconds, whereas almost all the flagella from cells of the two other species are released after such treatment.

b Cytoplasmic tubules in cells of *T refringens* seem to be less fragile than tubules in cells of *T phagedenis* and *T vincentii*.

c Cells of *T refringens* are less resistant to treatment with sodium deoxycholate than cells of the two other species. After treatment on grids for 30 seconds with 1 per cent sodium deoxycholate, flagella, cytoplasmic tubules, cellular debris and flakes of varying size of the outer surface layer of the cell wall

TABLE 3 *Test Levels for Equal Wavelengths by One-Way-Analysis of Variance and by Wilcoxon Test*

Test for equal wavelength	One-way-analysis of variance	Wilcoxon test
All strains	<0.05 %	
T phagedenis and *T vincentii*	6.2 %	5 %
T phagedenis and *T refringens*	1 1 %	2-5 %

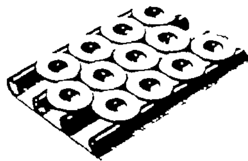


Fig. 31 The photograph shows model of the regularly structured surface layer of *T refringens* showing the characteristics described in the text.

seem to be the only remains of the majority of *T refringens* cells, whereas the cytoplasmic bodies of *T phagedenis* and *T vincentii* cells appear to be far less affected even after treatment for 2 minutes.

T phagedenis and *T vincentii* may be distinguished according to the wavelengths of the cells, as mentioned above The surface layer on untreated cells of *T phagedenis* does not in any instance show evidence of a substructure, whereas an indication of a substructure in this layer can be found on a few unfixed cells of *T vincentii* However for both species a substructure of the surface layer becomes evident in preparations of cells treated with *Myxobacter* AL-1 enzyme 1 The same type of pattern was seen on two micrographs of *T phagedenis* cells treated in suspension with Teepol. The pattern of the substructure appears to be similar to that observed in the surface layer of cells of *T refringens*

An idea of how the pattern of this regularly structured surface layer may be resolved by electron microscopy of negatively stained cells is outlined in Fig. 31 The rings are in close apposition to the parallel fibrils. The small less electron dense region often observed in the centre of the rings might be small projections which penetrate the layer of heavy metal salt normally present within the ring in negatively stained preparations.

The zigzag lines found in preparations of *T phagedenis* cells treated with AL-1 enzyme resemble the helices isolated from *Spirochaeta stenostrepta* (6) However the width of the zigzag lines is 11 nm, whilst the diameter of the helices was found to be 20–25 nm.

Thin sections reveal differences in width and density of the outermost cell wall layer of the three species studied The surface layers of cells of *T refringens* and *T phagedenis* have the same width, but the layer on *T phagedenis* cells is more electron dense than that on cells of *T refringens* The surface layer of *T vincentii* cells is 3–4 times as broad but has the same electron density as that found on cells of *T refringens*

Moureau & Grantini (12) studied *T minutum T calligyrum, T phagedenis* and *T refringens* in the electron microscope and concluded that the results obtained justified the classification of these strains as four different species. The main differences they observed between *T refringens* and *T phagedenis* were that *T refringens* had conical ends and showed no distinct outer membrane, while *T phagedenis* had blunt ends and a distinct outer membrane. These results are corroborated by the present study

Pillot (14) tested the antigenic relationship of *T phagedenis* and *T vincentii to T pallidum T minutum* and *T reiter* From the results it can be deduced that *T phagedenis* and *T vincentii* showed different degrees of reactivity towards the three species mentioned.

The strains of *T phagedenis* and *T vincentii* studied by *Moureau & Grantini* (12) by *Pillot* (14) and *Pillot & Ryter* (15) are the same as those used in the present study

Király et al. (10) have measured the content of some fatty acids in i.a *T refringens* and *T phagedenis.* They only found a difference in the percentage of unsaturated fatty acids in the two treponemes (10 per cent in *T phagedenis* versus 35 per cent in *T refringens*) The strains of *T refringens* and *T phagedenis* investigated were obtained from the collection of Institut Pasteur (personal communication) and are probably the same as those used for the present investigation.

Smibert (20) studied the production of alcohols and acids by fermentation of glucose by different treponemes. According to his results *T. phagedenis* and *T. vincentii* differ clearly and *T. refringens* differs slightly from *T. vincentii*. Unfortunately the paper does not state the origin of the strains used for the study.

A comparison of the results of the present study with those of *Moureau & Giuntini* (12), *Smibert* (20) and *Király et al.* (10) indicates that the investigated strain of *T. refringens* does belong to a species different both from *T. phagedenis* and *T. vincentii*. In addition when the results are compared with those of the immunofluorescence study of *Pillot* (14) it seems justifiable to conclude that the strains of *T. phagedenis* and *T. vincentii* also belong to different species.

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APPLICATION OF INDIRECT IMMUNOFLUORESCENCE, INDIRECT HAEMAGGLUTINATION AND POLYACRYLAMIDE-GEL ELECTROPHORESIS TO HUMAN T-MYCOPLASMAS

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An indirect immunofluorescence technique using unfixed colonies is applied to human T mycoplasmas. The method is found to possess the same degree of specificity as the growth-inhibition test and to be well suited for the identification and classification of T-mycoplasmas. The sensitivity is too low for the detection of antibodies in patient sera. A modification of the indirect haemagglutination test applicable to T-mycoplasmas was less specific, but owing to its sensitivity it can be recommended as a supplement to the metabolic inhibition test in studies of antibodies in patient sera. The polyacrylamide-gel electrophoresis appeared to be unsuitable for the identification and classification of human T-mycoplasma serotypes.

The metabolic inhibition test described by *Purcell et al.* (16) in 1966 was for several years the only technique available for serological studies of T mycoplasmas (6, 7, 17). In 1970 a preliminary report on the application of the growth inhibition, indirect immunofluorescence and indirect haemagglutination tests to T-mycoplasmas was published by *Black* (1). The growth inhibition test was later described in detail (2). In 1970 *Lin & Kass* (10) described an immune inactivation test suitable for studies of T-mycoplasmas (11, 12).

In addition to serological tests, attempts have been made to identify and classify T mycoplasmas by polyacrylamide-gel electrophoresis (19, 22).

The purpose of this report was (1) to describe the application of the immunofluorescence technique for identification and classification of human T-mycoplasmas, (2) to describe the application of the indirect haemagglutination test to human T mycoplasmas, (3) to compare the two tests with each other and with the growth inhibition and metabolic inhibition tests with respect to sensitivity, specificity and suitability in sero-epidemiological studies of non-gonococcal urethritis, (4) to determine the possible use of polyacrylamide gel electrophoresis in the identification and classification of serotypes of human T mycoplasmas.

MATERIAL AND METHODS

Organisms. Representatives of eight serotypes of human T-mycoplasmas (2) together with 22 strains isolated from human clinical material in this laboratory were employed.

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Preparation of antisera. Three to five albino rabbits were immunized with each of the eight serotypes by the method described by *Black* (2) Before use, all sera were absorbed with the medium (1 1) used for the preparation of antigens by incubation at 37 C for 1 hour and at 4 C for 6 hours. Separation was done by centrifugation at 4,000 rev/min for half an hour in an Ecco 258 centrifuge.

Patient sera. Samples were taken in the acute phase and two to three weeks later in the convalescence phase from T-mycoplasma positive patients suffering from non-gonococcal urethritis. None of the patients were treated with antibiotics during the sampling period.

Conjugation of serum. The method of *Nairn* (15) was followed, as described in detail by *Rosendal & Black* (20) The ratio of fluorescein isothiocyanate (FITC) to protein ranged from 2.5 to 4.5 For the indirect immunofluorescence a commercial horse anti-rabbit immunoglobulin (pk 17–4 F4) conjugated with FITC was used (Central laboratorium van de Bloedtransfusiedienst an het Nederlandsche Roode Kruis)

Unfixed colonies for immunofluorescence The T-mycoplasmas were grown on a modified Shepard medium (S-medium) consisting of trypticase-soy broth (Baltimore Biological Laboratories) 3 per cent, Ion agar No. 2 (Oxoid) 1 1 per cent supplemented with horse serum 20 per cent, yeast extract (baker's yeast) 10 per cent, and sodium penicillin 1,500 I U./ml. The pH was adjusted to 6.0. The plates were incubated in 90 per cent atmospheric air + 10 per cent CO at 37 C for 3 days unless otherwise stated.

Agar blocks cut in geometric figures and approximately ½ × ½ cm in size were used for immunofluorescence staining of unfixed colonies.

Fixed colonies for immunofluorescence. Hot water fixation of colonies was performed by the method of *Clark et al.* (4) as modified by *Rosendal & Black* (20)

Indirect and direct immunofluorescence (IDIF) The indirect technique has previously been described by *Black* (1) and *Rosendal & Black* (20) The agar blocks placed on slides were first incubated with a drop of serum dilution at 20 C for 30 min in a moist chamber After washing them twice in PBS (pH 7.2) for 10 min, the blocks were incubated with a drop of conjugated anti-rabbit globulin at 20° C for another 30 min in a moist chamber They were then again washed twice in PBS (pH 7.2) for 10 min, and for 1 min in distilled water Finally the blocks were again placed on slides and read as described below An agar block incubated with saline 0.9 per cent instead of serum was used as a negative control.

Direct immunofluorescence was carried out as described by *Rosendal & Black* (20) After incubating the agar blocks with one drop of conjugate or conjugate dilution at 20 C for 30 min in a moist chamber the blocks were washed twice for 10 min in PBS (pH 7.2) The blocks were then placed on slides and read.

Microscopy Fluorescence was studied under a Zeiss Standard (RA) microscope equipped with an Osram HBO mercury lamp and an incident-illumination attachment. The intensity of fluorescence was designated as 0 + ++ +++ viz. 0 for none and +++ for strong fluorescence. Titres were expressed as the reciprocals of the highest dilution giving +++ fluorescence.

Indirect haemagglutination (IHA) The IHA test was performed as described by *Kraggaard-Jensen* (9) To control the absorption of the hyperimmune rabbit antisera with S-medium they were tested against formalinized sheep-blood cells (RBC) coated with S-medium. If any reaction occurred, the absorption was repeated until the control was negative.

The organisms used for antigens were grown in 5-litre aliquots of S-medium. After being harvested, they were washed twice in PBS (pH 7 2) and finally resuspended in PBS (pH 7.2) with 2 per cent inactivated normal rabbit serum absorbed with RBC to give a 250-fold concentration of the original volume. The suspension was then sonicated in a Branson Sonifier (S-75) at 20 kc/min for 10 periods of 1 min on ice, and stored at –20 C until used. The antigens were standardized as described (9) and 2 units were used for the titration.

Formalinized RBC sensitized at pH 5.5 were used because fresh RBC were agglutinated by the T-mycoplasma antigens.

Growth inhibition (GI) The GI test was performed as described by *Black* (2)

Metabolic inhibition (MI) The MI test was performed as described by *Purcell et al.* (16). According to our experience this test is very reliable variations in repeated determinations never exceeding one titre step.

Polyacrylamide-gel electrophoresis. Each T-mycoplasma strain was grown in 5 l of S-medium at 37° C for 14 hours and harvested in a Sorvall RB centrifuge with continuous flow at 20,000 rev/min After being harvested the sediment was washed twice in PBS (pH 7.2) resuspended in 5 ml PBS (pH 7.2) and dialysed against distilled water for 24 hours. The protein content in each preparation was determined according to *Lowry et al.* (14) The preparations were lyophilized in 2-ml amounts.

Before electrophoresis, the lyophilized material was dissolved in 0.25 ml phenol-acetic acid-water (2 1-0.5) per mg protein

The polyacrylamide-gels were composed as described by *Razin et al.* (19) Glass tubes with an inner diameter of 6 mm and a length of 10 cm were filled to 7 cm with gel. After polymerization at room temperature, 0.1 ml of the soluble protein was placed on top of the gel and electrophoresis

TABLE 1 *Direct and Indirect Immunofluorescence Titres of Four Hyperimmune Sera Using Unfixed Colonies*

Antiserum	Direct method	Indirect method
1	40	320
2	10	40
3	20	160
4	10	80

was carried out at room temperature in a Canalco equipment at 5mA per gel for 75 min.

The gel was removed from the tubes and stained for 1 hour in 1 per cent Amido Black in 7 per cent acetic acid, destained in 7 per cent HAc and stored in 7 per cent HAc.

All serological and electrophoretic experiments in this study were carried out at least in duplicate.

RESULTS

Direct and indirect immunofluorescence of unfixed colonies. The sensitivity and specificity of direct and indirect immunofluorescence were compared using unfixed colonies as antigen. The sensitivity was expressed as the homologous titres indicated in Table 1 Four sera were compard. It applies to all four that the indirect method showed a 4- to 8-fold higher titre than the direct. In addition, the background fluorescence was much more pronounced if the direct method was used. To demonstrate the specificity four sera were tested against the eight serotypes of human T-mycoplasmas. As seen from Table 2, the specificity of the two methods appears to be equally high when the optimal dilution is used, i.e. the highest dilution of antiserum and conjugates that gives maximum fluorescence. Only the well-known reaction between antiserum against serotype II and T-mycoplasma serotype V (1 2, 6) was seen.

As the indirect method proved to be more sensitive and just as specific as the direct, and as the preparation of conjugated antiserum is laborious and requires a considerable amount of potent antiserum, only the indirect method was employed in the part of the study that is reported below

Indirect immunofluorescence of hot-water fixed and unfixed agar colonies. The indirect immunofluorescence titres of four hyperimmune sera using hot water-fixed and unfixed agar colonies were employed to compare the sensitivity of the test with the two preparations of antigen. As seen from Table 3 two of the sera showed slightly higher titres if unfixed colonies were used and two showed the same titres using either antigen preparation. The specificity was compared by testing three hyperimmune sera against hot-water fixed colonies and unfixed colonies of the eight serotypes. The results are shown in Table 4 Using fixed colonies, it applies to most antigens that heterologous fluorescence of varying intensity was seen whereas only the one-way cross-reaction between antiserum against serotype II and T mycoplasma serotype V was seen if unfixed colonies were used.

TABLE 2. *Cross-titration in Direct and Indirect Immunofluorescence Using Unfixed T-mycoplasma Colonies*

Antiserum against serotype No.	IMF	Serotype No. I	II	III	IV	V	VI	VII	VIII
I	Direct	+ + +	–	–	–	–	–	–	–
	Indir	+ + +	–	–	–	–	–	–	–
II	Direct	–	+ + +	–	–	+ + +	–	–	–
	Indir		+ + +	–	–	+ + +	–	–	–
III	Direct	–	–	+ + +	–	–	–	–	–
	Indir		–	+ + +	–	–	–	–	–
VII	Direct		–	–	–	–	–	+ + +	–
	Indir	–	–	–	–	–	–	+ + +	–

TABLE 3 *Indirect Immunofluorescence Titres of Four Hyperimmune Sera Using Unfixed and Hot-water-fixed Colonies*

Antiserum	Unfixed colonies	Hot-water-fixed colonies
1	320	80
2	40	40
3	160	80
4	80	80

Therefore hot water fixation of the T mycoplasma colonies cannot be recommended and was not used in the subsequent studies. Unfixed colonies could still be used as antigens after storage at 37 C for 20 days and at 4 C for 1 month in a moist chamber. After staining the unfixed colonies retained their fluorescence for more than 6 months if stored at 4 C in a moist chamber.

Indirect haemagglutination. The homologous reactions of eight hyperimmune sera were used for comparison of the IHA, indirect IMF, MI and GI tests with respect to their sensitivity (Table 5).

Good correlation was found only for sera Nos. 4 and 8, both showing a high content of antibodies as measured by all four tests. The other sera constantly revealed MI titres between 1,024 and 10,240, whereas either one or two of the other tests showed no or only weak reactions. The MI test must be regarded as the most sensitive, followed by the IHA, IMF and GI tests in that order. To compare further the sensitivity of the four tests, paired sera taken out at 2 week intervals from 10 patients with T-mycoplasma associated non-gonococcal urethritis (NGU) were tested against the eight serotypes of human T mycoplasma. The indirect immunofluorescence and the GI test showed no reaction with any of the 20 sera. Accordingly only the results of the MI and the IHA tests are listed in Table 6, together with the serotypes of T mycoplasmas isolated from the NGU patients. The isolates were typed by the GI test after cloning. It is seen that both tests can be used for detecting antibodies in patient sera, but no correlation between the results obtained by the two methods could be demonstrated. For example, serum No. 6 is the only one showed a significant rise (8-fold) in the homologous MI titre but no change in the IHA titre. The homologous IHA titre of serum No. 8 increased from 4 to 16, but so did all the heterologous titres, and some of them even more than 4-fold. The low specificity of IHA antibodies against serotype V is also demonstrated in Table 8. The MI titres were low and showed no rise. Serum No. 9 showed an 8-fold fall in homologous MI titre, but no changes in the IHA titre, whereas an 8-fold fall in heterologous IHA titre was seen in serum No. 3 without any significant change in the MI titre. Rises in only the heterologous titres were seen in sera Nos. 1 and 7 as measured by the MI test.

The rest of the sera showed either constant titres or a fall in heterologous titres as determined by one or both of the two methods.

The specificity of the indirect IMF and the IHA tests was studied and compared by cross-titrations. Using the eight human sero-

TABLE 4 *Cross-titrations in Indirect Immunofluorescence Using Unfixed and Hot-water-fixed Colonies*

Antiserum against serotype No.	Colony preparation	T-mycoplasma serotype I	II	III	IV	V	VI	VII	VIII
I	fixed	+ + +	−	+ +	+	−		+ +	+
	unfixed	+ + +	−	−	−	−		−	−
II	fixed	−	+ + +	+	+	+ + +	+	+ + +	+ +
	unfixed	−	+ + +	−	−	+ + +		−	
III	fixed	+	+ +	+ + +	+	+ +	+	+	+ +
	unfixed	−	−	+ + +	−	−		−	−

TABLE 5. *Eight Hyperimmune Sera Tested by GI MI IMF and IHA*

Serum No.	GI mm	MI Titre	IMF Titre	IHA Titre
1	0	10,240	320	640
2	0	1 024	40	1,280
3	5	10,240	40	160
4	8	16,384	160	2,560
5	4	8,192	320	80
6	3	8,192	40	1,280
7	0	10,240	320	1,280
8	4	163,840	160	10,240

types and their homologous antisera, the results could be compared with those obtained by the GI and MI tests, which have been published in a previous paper (2) and therefore are not shown here. As appears from Table 7 the indirect immunofluorescence with unfixed colonies was found to possess a specificity comparable to that of the GI test performed at 27 C, i.e. slightly more specific than the MI test and much more specific than the IHA test. A one-way cross-reaction between antiserum against serotype II and T mycoplasma serotype V was seen. Antiserum against serotype I reacted weakly with serotype II and so did antiserum against serotype VIII with serotypes IV and VII.

The results obtained by the IHA are listed in Table 8. The relatively high homologous titres made it possible to differentiate between the eight serotypes, but several one-way cross-reactions occurred, especially with antiserum against serotype V All the antisera reacted with from one to six of the heterologous antigens, mostly to a lower titre, but antisera VII and VIII with T mycoplasma serotypes VI and IV respectivly to the same titre as the homologous antigens.

To investigate further the specificity of the indirect IMF technique, it was used for the identification of 22 cloned isolates of human T mycoplasmas. All strains were identified as the same serotype as that previously found by the GI test. In addition, five mixed cultures were studied. The different serotypes could easily be identified by their homologous sera. If only relatively few colonies were present on the agar block, it was often possible to pick up ad subculture the different serotypes direct from the agar blocks used for the IMF

The IHA is seen to be less specific than both the MI, IMF and GI tests.

Polyacrylamide-gel electrophoresis To compare further the eight serotypes of human T mycoplasmas together with three fresh isolates, all identified serologically as serotype IV they were studied by polyacrylamide-gel electrophoresis. All the organisms showed a very similar pattern, the number of lines varying from 11 to 15 It was not possible to distinguish between intra- and interserotype differences as judged from this limited study

DISCUSSION

The investigations reported have demonstrated that both the immunofluorescence and the IHA techniques can be applied in the study of human T mycoplasmas.

In agreement with the findings obtained by *Rosendal & Black* (20) the indirect immunofluorescence method on unfixed colonies was found to be preferable to the direct method, because it seems to be more sensitive, possesses the same degree of specificity gives no background fluorescence, and requires only one conjugated serum.

Relatively low specificity of immunofluorescence using hot water fixed colonies has also been described for other mycoplasmas (20) A possible explanation could be that a partial denaturation of antigen occurs during fixation, as already advanced by *Chanock et al.* (5) The use of unfixed colonies seems to give a higher sensitivity as was also noticed in the case of canine mycoplasmas (20) Although fixed colonies can be stored for a very long period of time before use (15) the one month storage period applicable in the case of the unfixed T-mycoplasma colonies should meet the requirements for most purposes. The breakage of colonies as a result of washing as observed in other mycoplas-

TABLE 6. *Occurrence of Antibodies in Paired Sera from 10 Pat*

Patient sera	I		II		III		T-mycopla IV	
	MI	IHA	MI	IHA	MI	IHA	MI	IHA
1	16	8	4	8	8	8	8	4
	8	8	4	8	8	8	8	4
2	4	8	4	8	4	4	2	8
	2	4	2	2	2	4	2	2
3	4	4	2	2	4	2	32	2
	8	4	2	4	2	4	32	4
4	4	<2	4	<2	8	2	32	2
	4	<2	2	<2	8	2	16	<2
5	4	<2	4	<2	4	<2	8	<2
	4	2	2	2	4	<2	4	2
6	2	2	<2	2	4	2	4	2
	<2	<2	2	<2	2	<2	8	<2
7	4	2	<2	2	2	2	8	<2
	2	<2	<2	<2	4	<2	2	2
8	2	4	2	2	4	2	8	2
	2	16	2	64	4	32	4	16
9	*64*	2	<2	2	4	4	8	<2
	8	2	<2	<2	4	<2	2	<2
10	*8*	8	<2	8	8	8	8	8
	16	4	<2	4	4	4	2	4

mas (20) was not seen in the case of the T mycoplasmas.

No correlation between the presence of antibodies in hyperimmune sera could be demonstrated, as measured by the MI GI, indirect IMF and IHA tests. As a rule MI antibodies were those most easily obtained in high titres. As regards other human mycoplasmas similar results have been obtained in studies of patient sera (5 18, 21)

TABLE 7 *S rological Relationships Between Eight T-mycoplasmas Determined by the Indirect IMF Using Unfixed Colonies as Antigen*

T-myco-plasma serotypes	Antiserum against serotypes I	II	III	IV	V	VI	VII	VIII
I	*320*	<2	<2	<2	<2	<2	<2	<2
II	20	*40*	<2	<2	<2	<2	<2	<2
III	<2	<2	*40*	<2	<2	<2	<2	<2
IV	<2	<2	<2	*160*	<2	<2	<2	10
V	<2	40	<2	<2	*320*	<2	<2	<2
VI	<2	<2	<2	<2	<2	*40*	<2	<2
VII	<2	<2	<2	<2	<2	<2	*160*	40
VIII	<2	<2	<2	<2	<2	<2	<2	*320*

…ing from NGU as Measured by the MI and IHA Techniques

…ype V MI	V IHA	VI MI	VI IHA	VII MI	VII IHA	VIII MI	VIII IHA	Serotype of isolates from the pts.
8	8	8	8	2	4	4	4	IV
4	4	8	16	16	4	4	2	
4	8	4	8	8	8	*4*	*16*	VIII
2	4	2	4	4	4	*4*	*8*	
4	4	4	4	4	4	4	*16*	IV
2	4	2	4	2	4		2	
8	<2	8	<2	16	<	4	<2	III
4	<2	4	<2	32	<2	4	<2	
4	<2	4	<2	<2	<2	2	8	III
2	2	2	2	<2	<2	2	16	
<2	2	<2	2	*32*	2	2	<2	VII
<2	<2	<2	<2	*256*	<2	4	<2	
<2	2	<2	<2	*8*	2	<2	<2	III
<2	2	8	<2	*8*	<	2	<2	
2	*4*	2	2	2	2	4	4	V
4	*16*	<2	16	4	16	4	32	
4	2	4	2	<2	2	4	<2	I
2	<2	2	<2	<2	2	2	<2	
2	8	2	8	32	8	4	16	I
2	4	<2	4	32	4	2	8	

A comparison of the IMF IHA, MI and GI tests showed that the GI test performed at 37 C is the most specific test, whereas the GI test performed at 27 C and the indirect IMF are slightly less specific, followed by the MI test. The IHA test was found to be relatively unspecific. These findings are in over-all agreement with the conclusions drawn by *Purcell et al.* (18)

The previous establishment of eight serotypes of human T mycoplasmas (2) was confirmed by both IMF and IHA.

TABLE 8. *Serological Relationship Between Eight T mycoplasmas Determined by the IHA*

T-myco-plasma serotypes	Antiserum against serotypes I	II	III	IV	V	VI	VII	VIII
I	*320*	<10	<10	40	40	40	<10	<10
II	80	*640*	<10	<10	160	40	<10	20
III	40	<10	*1280*	80	40	<10	<10	<10
IV	<10	<10	<10	*320*	<10	<10	<10	320
V	<10	40	<10	40	*320*	<10	<10	<10
VI	<10	<10	80	<10	40	*160*	160	<10
VII	<10	<10	<10	<10	80	80	*1280*	<10
VIII	<10	<10	<10	<10	160	20	<10	*10240*

The MI and IHA tests are the most sensitive and the only ones that can be recommended for detecting antibodies against T mycoplasmas in human sera. However no correlation between the results obtained by the two methods was seen. The four serological tests were not compared with the immune inactivation test (10) because of difficulties experienced in reproducing the test in our laboratory

Among 10 paired sera from NGU patients, only two showed a significant rise in homologous titres, one demonstrated by each of the two methods. In some sera, a rise in antibody titre against serotypes other than that isolated from the patient was demonstrated. The explanation may be that these patients originally harboured a mixed flora of T mycoplasmas, which is known to occur (12) but only one serotype was selected by the cloning procedure performed before the identification by the GI test. If the IMF technique had been used for identification, this could have been done on the primary isolation plates mixed cultures would have been detected and better information of the epidemiology obtained.

Serological response to T mycoplasmas in NGU patients has previously been described by *Ford* (6) and *Jansson et al.* (8) who used the MI test and the IHA test, respectively whereas *Purcell et al.* (17) failed to demonstrate a significant rise in titre in 20 paired sera using T 960 as antigen in the MI test. From this study it is seen that it is advisable to employ all known serotypes as antigens and to use both the MI and the IHA test Until the changes in antibody level and globulin classes during T mycoplasma infection have been studied in detail only a significant rise in antibody titre can be considered as indicating infection.

Gel-electrophoretic analysis of the human T-mycoplasmas was found to be unsuitable for the differentiation between and identification of the serotypes. This is in accordance with previous investigations (19 22)

It can be concluded that the indirect immunofluorescence using unfixed colonies on agar blocks is comparable to the GI test in suitability for serological identification and classification of human T mycoplasmas and as regards primary identification it is even superior The IHA can be used as a supplement to the MI test if antibodies in patient sera are to be detected, but it cannot be recommended for identification and classification.

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THE SYNTHESIS OF PHOSPHOLIPASE C BY *BACILLUS CEREUS*

II A Screening Method for Mutants

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A sensitive method allowing rapid screening for the presence of phospholipase A or C in solutions or the production of these enzymes by bacteria is described. By this method, a phospholipase C hyper-producing mutant has been detected.

Phospholipase C (EC 3 1 4.3) is an exo-enzyme produced by some members of the family Bacillaceae. Its physiological role is not well-established, but the enzyme is widely used as a tool in membrane studies. We have reported a method for the purification of phospholipase C from *Bacillus cereus* (ATCC 10987) (2) and shown that the synthesis of the enzyme can be inhibited by a low molecular component of certain batches of Brain Heart Infusion (3) In order to increase the yield of enzyme and to study the physiological role and biosynthetic regulation of the enzyme, we have investigated phospholipase C synthesis mutants.

We report here a convenient screening method for such mutants. It is also applicable to phospholipase A. By this method we have detected a hyper producing phospholipase C mutant.

MATERIALS AND METHODS

Bacillus cereus ATCC 10987 and various mutants derived from this strain were used. Liquid cultures were maintained as described (3) and growth estimated by optical density at 600 nm. Mutagenesis with nitrosoguanidine was carried out as described by *Adelberg* (1)

Agar plates were made of the dialysate (low molecular weight fraction) of Nutrient Broth (Oxoid) with 1 per cent agar (Difco) The dialysis bag contained 26 g Nutrient Broth per litre which was dialysed against an equal volume of distilled water for 14 h at 4 C.

In some cases, Brain Heart Infusion (Oxoid) was used instead of Nutrient Broth at a concentration of 74 g per litre in the dialysis bag.

In both cases, 5 per cent (v/v) Colbeck EY Broth (Difco, Batch No. 0234–72) was added to the agar as a phospholipid source.

Phospholipase C was purified as described (2) and its activity measured according to *Ottolenghi* (4) with the following modification Colbeck EY Broth was diluted (1 1) with veronal buffered saline (pH 7 4) containing 1 mM $ZnCl_2$. One enzyme unit was defined as an increase in turbidity (decrease in transmission) of 100 per cent per min at 27 C. Phospholipase A from *Vipera russelli* venom (Sigma) was used as obtained.

Antisera were raised in rabbits by monthly subcutaneous injections of about 300 µg of purified phospholipase C mixed with Freund's complete adjuvant. The immunoglobulin fraction was isolated by ammonium sulphat precipitation and DEAE Sephadex batch treatment. Immunodiffusion was carried out as described (5)

RESULTS AND DISCUSSION

The Screening Method

The method uses the well-established increase in turbidity observed during phos-

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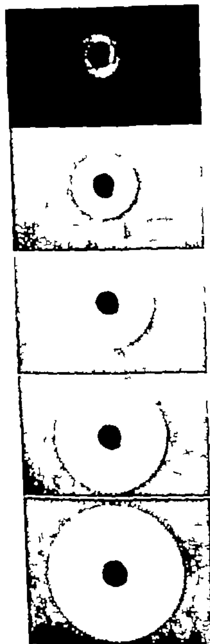


Fig. 1 Phospholipase C on agar plate containing 5 per cent Colbeck EY Broth.
1 μg PLC (50 μl) was added to the well (diameter 6 mm). The plate was incubated at 37 C and photographed after 1 2, 4 6 and 11 h.

pholipase C catalysis of diglyceride formation from a suitable phospholipid suspension (4) If the phospholipid source was included in the agar plates, phospholipase C caused the formation of a turbid zone around the point of application of the enzyme or the colony of enzyme producing bacteria (Fig. 1)

If 50 μl of a solution of purified phospholipase C (20 μg/ml) was added to a well (diameter 6 mm) in the agar plate a turbid zone was seen after 30 min at 37 C. The area of the zone increased linearly with time over about 11 h (Fig. 2) Similar results were obtained using enzyme generated *in situ* by a colony of bacteria.

The area was plotted against the amount of enzyme in double logarithmic plots to obtain straight lines suitable as standard curves. 5 to 10 ng per 50 μl were easily detectable. Being both rapid and sensitive the method is well suited for analysis of for instance, column effluents.

Phospholipase A gave a similar turbid zone after 30 min at 37 C if 20 μg (0 1 unit) of a commercial enzyme preparation was used in wells of diameter 2 mm. The *B cereus* strains in which synthesis of phospholipase C was inhibited by BHI-medium gave normal growth but no turbid zone on BHI-agar plates

A Hyper producing Mutant

After mutagenesis with nitroguanidine, about 6 000 colonies were screened for phospholipase C production on agar plates. One mutant (ATCC 10987 AB-1) was found which produced a larger zone on the agar plates in a given time. Phospholipase C was purified from medium of liquid cultures (dialysed Nutrient Broth) of this strain. The new strain yielded 5-times more enzyme than the parental strain. The specific activity of the purified enzyme was the same for both strains (about 420 units/mg) In addition, a reaction of complete identity was obtained when the precipitation line of such enzyme preparations against the anti-phospholipase C antiserum was compared to that of enzyme produced by the parental strain. The mutation was therefore not in the gene coding for the polypeptide. Neither he parental strain

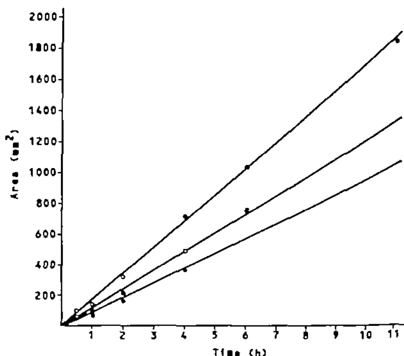


Fig 2 Area of turbid zones as a function of time. Various amounts of phospholipase C were added to wells in agar plates containing 5 per cent Colbeck EY Broth. The plates were incubated at 37 C.
10 μg PLC (50 μl) o——o
1 μg PLC (50 μl) □——□
0.1 μg PLC (50 μl) •——•

nor this mutant were inhibited by BHI medium in their synthesis of phospholipase C.

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BIOCHEMICAL AND SEROLOGICAL PROPERTIES OF *STREPTOCOCCUS MUTANS* FROM VARIOUS HUMAN AND ANIMAL SOURCES

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The main part of strains of *Streptococcus mutans* isolated from the present Danish material of blood from patients with subacute endocarditis and from human teeth belonged to two of five serotypes established by *Bratthall* viz. type c and type e. Two new types were established type f and type g. Strain SL-1 seems to constitute a distinct type. Strains of serotypes a and b have not been isolated in Denmark, and strains of serotypes d, g and SL have been isolated from teeth only. The registered differences in biochemical behaviour warrant a proposal of a subdivision into three biotypes.

The first description of *Streptococcus mutans* was given by *Clarke* in 1924 in a paper entitled "On the bacterial factor in the aetiology of dental caries" (9). In 1928 *Abercrombie & Scott* (1) reported on the isolation of the same organism from a case of subacute endocarditis. *S. mutans* has not been mentioned in the literature dating back to the Thirties and the Fourties and, in the 7th edition of Bergey's Manual 1957 the description of this organism, a description which appeared for the first time in the 6th edition, 1948, is no longer retained.

In the Fifties and early in the Sixties, odontologists focused their attention on cariogenic streptococci (15, 16, 21) and late in the Sixties, cariogenic streptococci identified as *S. mutans* were isolated from human carious lesions and human dental plaques and blood (7, 8, 14, 18, 24) and it was realized that *S. mutans* is a characteristic and "clearly defined distinct species" (13).

The strains originally described by *Clarke* have been lost, but a neotype strain, isolated from the deepest layer of human carious dentine, has been proposed by *Sims* viz. NCTC 10449 (27).

Serological studies of strains from human and animal sources have by now revealed five different antigenic types designated a, b, c, d and e by *Bratthall* (2, 3, 4, 19, 24, 28, 33). *Coykendall* (11, 12) has demonstrated the existence of four genetic groups which paralleled four of *Bratthall's* five serotypes.

An examination of streptococci isolated from blood during the years 1959–1971 at the Statens Seruminstitut, Copenhagen, disclosed a marked frequency of *S. mutans* in patients with subacute endocarditis. The study of these strains, a series of strains of *S. mutans* from persons with carious lesions

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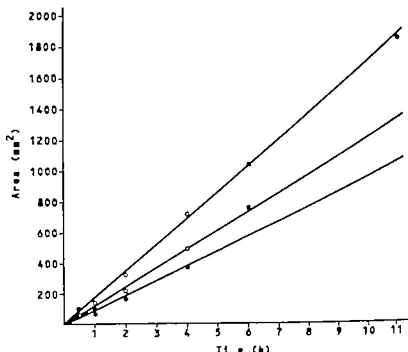


Fig 2 Area of turbid zones as a function of time. Various amounts of phospholipase C were added to wells in agar plates containing 5 per cent Oolbeck EY Broth. The plates were incubated at 37 C.
10 μg PLC (50 μl) ○——○
1 μg PLC (50 μl) □——□
0 1 μg PLC (50 μl): ●——●

nor this mutant were inhibited by BHI medium in their synthesis of phospholipase C

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The main part of strains of *Streptococcus mutans* isolated from the present Danish material of blood from patients with subacute endocarditis and from human teeth belonged to two of five serotypes established by *Bratthall*, viz. type c and type e. Two new types were established type f and type g. Strain SL-1 seems to constitute a distinct type. Strains of serotypes a and b have not been isolated in Denmark, and strains of serotypes d, g and SL have been isolated from teeth only. The registered differences in biochemical behaviour warrant a proposal of a subdivision into three biotypes.

The first description of *Streptococcus mutans* was given by *Clarke* in 1924 in a paper entitled "On the bacterial factor in the ætiology of dental caries" (9). In 1928 *Abercrombie & Scott* (1) reported on the isolation of the same organism from a case of subacute endocarditis. *S. mutans* has not been mentioned in the literature dating back to the Thirties and the Forties and in the 7th edition of Bergey's Manual, 1957 the description of this organism, a description which appeared for the first time in the 6th edition, 1948, is no longer retained.

In the Fifties and early in the Sixties, odontologists focused their attention on cariogenic streptococci (15 16 21) and late in the Sixties, cariogenic streptococci identified as *S. mutans* were isolated from human carious lesions and human dental plaques and blood (7 8, 14 18, 24) and it was realized that *S. mutans* is a characteristic and "clearly defined distinct species" (13).

The strains originally described by *Clarke* have been lost, but a neotype strain, isolated from the deepest layer of human carious dentine, has been proposed by *Sims* viz. NCTC 10449 (27).

Serological studies of strains from human and animal sources have by now revealed five different antigenic types designated a, b, c, d, and e by *Bratthall* (2, 3 4 19 24 28, 33). *Coykendall* (11 12) has demonstrated the existence of four genetic groups which paralleled four of *Bratthall's* five serotypes.

An enumeration of streptococci isolated from blood during the years 1959–1971 at the Statens Seruminstitut, Copenhagen, disclosed a marked frequency of *S. mutans* in patients with subacute endocarditis. The study of these strains, a series of strains of *S. mutans* from persons with carious lesions

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and dental plaques and some strains from other laboratories was undertaken in order to confirm and possibly add to the existing knowledge about serological and biochemical characteristics of *S mutans* and also in order to compare oral strains and strains isolated from blood to see whether certain serotypes occur at a higher frequency in subacute endocarditis than other serotypes.

MATERIAL AND METHODS

The 210 strains studied are listed in Table 1 together with the origin and the serotype determined by us. 180 of these strains were isolated at this institute 54 originated from the blood of 54 patients with subacute endocarditis, 59 from human dental plaques of 56 persons and 67 from human carious lesions in 58 persons.

The remaining 30 strains one of which is the neotype strain NCTC 10449 were isolated by others. These 30 strains comprise two strains from human blood, 17 from human dental plaques, three from human carious lesions and one from the human oral cavity Four strains were isolated from hamsters and three from teeth of rats.

Antisera were prepared by immunizing rabbits with streptococci grown in Todd-Hewitt broth for about 18 hours at 36 C. The culture was killed by heating at 60 C for 30 minutes, washed three times in saline and stored in saline in a concentration of 1 100 of the original volume with 0.5 per cent formalin added as preservative. The concentration of bacteria used for immunization was either 4×10^9 or 8×10^9 organisms per ml, and the course of immunization was either 4 weeks or 2–3 weeks, respectively The animals were given three successive injections of 0.5 ml the first week and of 1 ml the following weeks. The animals were not bled until a strong capillary precipitation reaction had developed. Bleeding generally took place 5 or 6 days after the last injection.

Acid extracts were made according to Lancefield, with 0.2 N HCl or with 0.066 N HCl (26) Formamide extracts were made according to Fuller using antigen obtained both from precipitation with acid ethanol, and from precipitation with acetone of the supernatant after acid ethanol precipitation. Interfacial ring precipitation was carried out in microtubes (diameter 1.5 to 2.0 mm) layering the extracts on the sera.

The fluorescent antibody test (FAT) was carried out as described by *Inga Lind* (22) Heat fixed preparations were introduced in repeated tests. Absorption was performed with tightly packed cells from a 20-hour Todd Hewitt broth culture killed at 60° C and washed twice in buffered saline. The absorbed sera could be stored at 4 C for at least 10 months without significant decrease in the intensity of fluorescence.

Fluid media were inoculated with two drops of an overnight Todd-Hewitt broth culture unless otherwise stated. Final pH was measured after growth in plain broth containing 1 per cent glucose.

Production of *dextran* was studied in tryptose Difco broth containing yeast extract, potassium acetate and 5 per cent sucrose. Dextran was detected by precipitation with pneumococcus type 2 antiserum (*Lund* MD Statens Seruminstitut) of the diluted supernatant (as a rule 1 10 or 1:100), or by precipitation with one volume of ethanol. Production of *slime* was examined on ovoid agar no. 2 containing 5 per cent sucrose. Production of *acetoine* was detected by the appearance of a reddish colour after shaking 2 ml of the culture (grown in buffered bacto-tryptose-yeast extract containing 0.5 per cent glucose for 4 days) with 1 ml of a 6 per cent alcoholic solution of α-naphtol and 0.4 ml potassium hydroxide (40 per cent)

Tolerance to *bile* (10 and 40 per cent ox bile) and *sodium chloride* (4 and 6.5 per cent) was studied by growth on ox agar containing 5 per cent defibrinated horse blood and the respective inhibitors. Tolerance to *tellurite* was studied on ox agar mixed at 80 C for 5 minutes with 10 per cent defibrinated horse blood and potassium tellurite (1.2500) *Growth at 10° C and 45° C* was observed in Todd-Hewitt broth. *L-arginine dihydrolase* was demonstrated by Nessler's reagent, and also by using the method of *Møller* (25) which, in addition, discloses decarboxylase activity. Hydrolysis of *starch* was tested on ovoid agar no. 2 containing 0.2 per cent soluble starch by flooding the plate with Lugol's iodine after 2 days of incubation. Hydrolysis of *sodium hippurate* was detected by the appearance of a crystalline precipitate after addition of one part of 50 per cent sulphuric acid to two parts of culture grown in plain broth containing 1 per cent sodium hippurate. Hydrolysis of *esculine* was detected by a blackening of ovoid agar no. 2 containing 0 1 per cent esculine and 0.05 per cent ferric citrate. Production of acid from fermentable substances was studied for 8 days in Difco phenol red broth base supplemented with 0.2 per cent bacto beef extract. The substances mentioned in the text (pages 364 366) and listed in Table 6 were tested. Glycerol was used in a concentration of 0.2 per cent, the remaining substances in a concentration of 1 per cent. Haemolysis was studied on ox agar containing 5 per cent defibrinated horse blood. Production of peroxide was investigated according to *Whittenbury* (32) Sensitivity to sulphathiazole (238 µg) was determined by the prediffusion method of *Thomsen* (29) The solid media were incubated in jars con-

TABLE 1 *List of Strains their Origin and Type Diagnosis*

Strain	Origin	Isolated by	Isolated from	Serotype
HS-1	*Fitzgerald*	*Fitzgerald*	Hamster	a
HS-6	*Fitzgerald*	*Fitzgerald*	Hamster	a
OMZ 61	*Guggenheim*	*Guggenheim*	Plaque (rat)	a
OMZ 49 (= E 49)	*Guggenheim*	*Fitzgerald*	Plaque (hamster)	a
NIDR 3720 (HS-6*)	*Colman*	*Fitzgerald*	Plaque (hamster)	a
AHT	*Zinner*	*Zinner*	Plaque (homo)	a
FA-1	*Fitzgerald*	*Fitzgerald*	Caries (rat)	b
BHT	*Zinner*	*Zinner*	Plaque (homo)	b
OMZ 71a	*Guggenheim*	*Guggenheim*	Plaque (rat)	b
OMZ 51x	*Guggenheim*	*Guggenheim*	Plaque (homo)	b
NCTC 10449*	Neotype	*Sims*	Caries (homo)	c
Ingbritt	*Kilian*	*Krasse*	Plaque (homo)	c
GS 5	*Colman*	*Gibbons*	Caries (homo)	c
FW293	*Colman*	*Colman*	Blood (homo)	c
MPK 1	*Colman*	*Carlsson*	Mouth (homo)	c
UTB 2	*Colman*	*de Stoppelaar*	Plaque (homo)	c
OMZ 70	*Guggenheim*	*Guggenheim*	Blood (homo)	c
50 strains	S.S.†	S.S.	Blood (homo)	c
46 strains	S.S.	S.S.	Plaque (homo)	c
46 strains	S.S.	S.S.	Caries (homo)	c
UTB 1	*Colman*	*de Stoppelaar*	Plaque (homo)	e+
B 2	*Bratthall*	*Carlsson*	Plaque (homo)	e
LM 7	*Bratthall*	*Gibbons*	Caries (homo)	e
3 strains	S.S.	S.S.	Blood (homo)	
2 strains	S.S.	S.S.	Plaque (homo)	e
7 strains	S.S.	S.S.	Caries (homo)	e
OMZ 175	*Bratthall*	*Guggenheim*	Plaque (homo)	f+
1 strain	S.S.	S.S.	Blood (homo)	f
7 strains	S.S.	S.S.	Plaque (homo)	f
1 strain	S.S.	S.S.	Caries (homo)	f
B 13	*Bratthall*	*Carlsson*	Plaque (homo)	d
OMZ 176	*Bratthall*	*Guggenheim*	Plaque (homo)	d
OIHI	*Bratthall*	*Carlsson*	Denture (homo)	d
RIV 10987‖	*Colman*	*de Moor*	Plaque (homo)	d+
RIV 10986	*Colman*	*de Moore*	Plaque (homo)	d+
RIV 10985	*Colman*	*de Moor*	Plaque (homo)	d+
TH 7	S.S.	S.S.	Caries (homo)	d
K 1	*Colman*	*Fitzgerald*	Plaque (homo)	g
OMZ 65	*Bratthall*	*Guggenheim*	Plaque (homo)	g
3 strains	S.S.	S.S.	Plaque (homo)	g
5 strains	S.S.	S.S.	Caries (homo)	g
1 strain	S.S.	S.S.	Plaque (homo)	Not typeable
7 strains	S.S.	S.S.	Caries (homo)	Not typeable
SL-1	*Coykendall*	*MacCabe*	Plaque (homo)	Related to d, g

+ apparently not typed earlier
*NCTC = National Collection of Type Cultures.
†S.S. = Statens Seruminstitut, Copenhagen.
‖RIV = Rijksinstituut voor de Volksgezondheid, Utrecht.

taining 10 per cent carbon dioxide. The incubation temperature was 36 C.

RESULTS

Antigenic Relationship Between S. mutans *and Streptococci Belonging to Different Lancefield Groups*

In precipitin tests, with antisera to streptococcus groups A to T and Fuller extracts of types a, b c, d, e, f and g of *S. mutans* antiserum to Lancefield's group E was the only serum which strongly precipitated extracts of *S. mutans* namely type e. In addition, one out of three antisera to group E was able to give a weaker precipitation with extracts of *S. mutans* of types c and f.

In precipitin tests with antisera to all types of *S. mutans* and Fuller extracts of representatives of streptococcus groups A to T strong precipitin reactions occurred with extracts of group E strains (K 129 K 131). Occasionally weaker reactions occurred with extracts of strains belonging to group F group K (strain Levy) *S. bovis* and *S. salivarius* (8606 and Packham). In addition, a single, very potent antiserum to a *S. mutans* type c strain precipitated extracts of group A and group D strains. Relatively specific precipitating antisera were chosen for further investigation.

Cross-absorptions of antisera to *S. mutans* type e and Lancefield group E left antibody residues for the homologous strain, which indicated that each type contained a distinct antigen. Thus, the Lancefield group E antigen is not identical with *S. mutans* type e antigen.

Antigenic Relationship Between Strains of S. mutans *(PRECIPITIN TEST)*

Using appropriate antisera it was possible to distinguish the five types a, b, c, d, and e established by *Bratthall* (3). Furthermore, two new types were found, *viz.*, one, comprising nine strains from blood and teeth and *Bratthall's* type x (strain OMZ 175) which we propose to name type f in keeping with the nomenclature of *Bratthall*, and another, comprising eight strains from teeth, strain

TABLE 2. *Precipitin Reactions with Strains of S. mutans*

Type of antigen*	Crude antisera from rabbits immunized with						
	FA–1 type b	NCTC 10449 type c	SE 3 type e	SE 11 type f	HS–1 type a	B 13 type d	OMZ 65 type g
b	+ + +	0	0	0	0	0	0
c	0	+ + +	(0/+)	(0/+)	0	0	0
e	0	0	+ + +	0	0	0	0
f	0	(0/+)	0	+ + +	0	0	0
a	0	0	(0/+)	0	+ + +†	+ + +	+ + +
d	0	0	(0/+)	0	+ + +	+ + +	+ + +
g	0	0	(0/+)	0	+ + +	+ + +	+ + +
SL–1	0	0	+§	0	+§	+ + +§	+ + +§

*Extraction by formamid 1) Antigen from acid ethanol precipitate.
Extraction by formamid 2) Antigen from acetone precipitate.
Extraction by HCl 3) Antigen from 0.2 N HCl.
†HCl extracts of some strains were precipitated only if extracted by 0.066 N HCl.
+ + + means strong precipitation within 5 minutes.
+ + and + mean precipitation within 10 and 15 minutes, respectively.
(0/+) means that some strains were weakly precipitated.
§means that precipitation occurred with HCl extracts only.

TABLE 3 *Immunological Cross-reactions of S. mutans in Precipitin Test with Absorbed Antisera*

Type of antigen*	Antiserum from rabbits vaccinated with								
	Strain HS-1 (type a) Abs. with culture of type			Strain B 13 (type d) Abs. with culture of type			Strain OMZ 65 (type g) Abs. with culture of type		
	d	g	SL-1	a	g	SL-1	a	d	SL-1
†	+ + +	+ + +	+ + +	0	+	+ + +	0	+ + +	+ + +
d	0	0	+ + +	+ + +	+ + +	+ + +	+ + +	0	+ + +
g	+ + +	0	+ + +	+ + +	0	+ + +	+ + +	+ + +	+ + +
SL-1			0			0			0
Factor	2, 3	3		4 5	5+		4	2	

For explanation of signs, see Table 2.
For explanation of factors, see text.
5 + means the presence of an additional antigen related to serotype a.
means not done.

OMZ 65 and strain K1 which we propose to name type g.

By use of the precipitin test, strains which belonged to types b, c, e and f could be typed by unabsorbed antisera, although *weak* cross-reactions sometimes occurred (Table 2) Strains which belonged to types a, d and proposed type g could be typed by means of absorbed sera only since all strains of these latter types gave rise to *strong* cross-reactions (Table 2) The common factor which gives rise to the cross-reaction of the three types (Table 2) is arbitrarily designated factor 1 (compare the schematically recorded for mulae (page 362) Results of precipitin test with extracts of strains of types a, d, and g and cross-absorbed sera are shown in Table 3 The common factor between type a and proposed type g, which remains in antiserum a and antiserum g by absorption with organ isms of type d, is designated factor 2. An antibody residue, specific for type a, which remains in type a serum after absorption with organisms of type g, is designated factor 3 The common factor between type d and type g, which remains in antiserum to type d and type g, absorbed with organisms of type a is designated factor 4. The antibody residues, *almost* specific for type d, which remain in type d serum after absorption with organisms of type g, is designated factor 5 Absorption of antiserum to type g with organisms of

TABLE 4 *Absorptions Necessary to Obtain Type-specific Sera*

Type of serum*	Strains used for immunization	Types and strains used for absorption
b	FA-1	f (SE 11) and S. salivarius (8606)
	NCTC 10449	(SE 3)
e	SE 3	c (NCTC 10449) and group E (K 129)
f	SE 11	e (SE 3)
	HS-1	g (OMZ 65) and d (B 13)
d	B 13	(HS-1) and g (OMZ 65)
g	OMZ 65	(HS-1) and d (B 13)

*Sera were conjugated before absorption.

type a and type d apparently depleted the serum for precipitins, and is not recorded in Table 3

Antigenic Relationship Between Strains of S. mutans *(FAT)*

All sera had to be absorbed in order to carry out a type determination with the FAT Several absorption experiments finally showed that type specific sera could be obtained as indicated in Table 4

The FAT with absorbed sera and strains of types a, d and g confirmed the antigenic formulae based on the results of the precipitin tests, but disclosed the existence of further antigens (Table 5)

Some of the type d strains gave a fluorescent reaction, although weak, with a factor which we call 6 (antiserum to OMZ 65 absorbed with types a and d) Some of the strains typed as g by the precipitin test were shown in FAT to contain an antigen related to but not identical with, the factor 5 specific for type d (antiserum to B 13 absorbed with types a and g) as absorption of anti-type d serum with such type g strains (TH) did not exhaust the serum. If however anti-type d serum was absorbed with type a, type g and *S salivarius* (8606) none of the type g strains exhibited fluorescence. Based on the FAT with absorbed sera, type d could thus be differentiated by the presence or absence of a factor 6 and strains of type g could be differentiated by the presence or absence of a factor related to factor 5 and *S salivarius.*

Schematically the formulae for types a, d and g may be expressed as follows

Type a 1 2, 3+
Type d 1 4 5+
Type d 1 4 5+ 6
Type g 1 2 4 6
Type g 1 2, 4 6, (5)
SL–1 4

The + indicates the presence of an additional antigen common to types a and d brackets indicate part of an antigen and dots indicate possible unknown antigens.

Strain SL–1 obviously carries only the factor 4 common for type d and type g and seems to constitute a distinct type. No antiserum, however was prepared with this strain. In the FAT strain SL–1 gave weak or no fluorescence with antiserum to type a (HS–1) whether unabsorbed or absorbed with strain B 13 Absorption of antisera to types a, d and g with strain SL–1 did not significantly reduce the content of precipitins and fluorescent antibodies (Tables 3 and 5)

Cultural and Biochemical Behaviour

Carbon dioxide favoured growth. Some strains would not grow at all except in the presence of carbon dioxide, *e.g* NIDR 3720, HS–6, OMZ 61 RIV 10985 RIV 10986 and TH 7 Growth on horse blood agar appeared as small white colonies, either irregular in shape or regular with a slime-like rim. Both forms adhered to the surface with a tendency to grow down into the medium. Most white forms split off larger colonies which were greyish and flat and never adherent to the medium. Such colony variants were not noticed to revert to the original form, even after repeated subcultures. This colony dissociation seemed to become more frequent after storage in semifluid agar The variant form retained the haemolytic activity and the biochemical and serological properties of the original form.

The dissociation took place in strains of serotypes b, c, e and f No dissociation on horse blood agar was observed in the case of strains of serotypes a, d, g and SL–1 Strains of these types appeared in a form resembling the flat variant form described above. One strain of type g, our number 144b, dissociated into a translucent slimy and an opaque non-slimy colony on sucrose agar presumably similar to strains described by *Edwardsson* (14) Sixteen out of 54 strains from blood were noted to exhibit varying degrees of β haemolysis on blood agar plates. One type c strain (Ingbritt) one type a strain (AHT) and all type d g and SL–1 strains gave rise to α-haemolysis.

All strains were non-motile, catalase-nega-

TABLE 5 *Immunological Cross-reactions of S mutants in FAT with Conjugated Absorbed Antisera Diluted 1 8*

Type of antigen	Antiserum from rabbits immunized with											
	Strain HS–1 (type a) Abs. with culture of type				Strain B 13 (type d) Abs. with culture of type				Strain OMZ 65 (type g) Abs. with culture of type			
	d	g	d+g	SL–1	a	g	a+g	SL–1	a	d	a+d	SL–1
	+ + +	+ + +	+ + +	+ + +	0	0–+ +	0	+ +/+ + +	0	+ +–+ + +	0	+ + +
d (B 13)	0	0–+	0	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	0	0	+ + + +
d (OIHI)	0	0–+	0	+ + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ +	+ +	+ + + +
g (OMZ 65)	+ + +	0	0	+ + +	+ + + +	0	0	+ + + +	+ + + +	+ + + +	+ +	+ + + +
g (TH)	+ + +	0	0	+ + +	+ + + +	+ +/+ + +	+–+ + +	+ + + +	+ + + +	+ + + +	+/+ +	+ + + +
SL–1	0	0		0	+ + +	0		0	+ + +	0		0
Factor	2, 3	3+	3		4 5	5+	5		4 6	2, 6	6	

Explanations of signs:
+ + + + = brilliant fluorescence.
+ + + = less brilliant fluorescence.
0–+ + + = variation in degree of fluorescence of bacteria in the same pure culture.
+/+ + = variation in degree of fluorescence of bacteria in different strains.
0 = no fluorescence.
3+ and 5+ = the presence of an additional antigen common to type a and type d.
= not done.

TABLE 6. *Differences in Biochemical and Cultural Behaviour*

Serotype	Strains	Number	Haemolysis	Arginine	Lactose	Melibiose	Raffinose	Salicin	Esculin	Inulin	Mannitol	Sorbitol	Peroxide	45 C
a	OMZ 49 OMZ 61 HS–1 HS–6	4	γ	−	+	+	+	+	+	+	+	+	−	−
	AHT	1	α	−	+	−	×	+	+	+	+	+	−	−
	NIDR 3720	1	γ	−	−*	−*	+	+	+	+	+	+	−	
b	FA–1 BHT OMZ 71a, OMZ 51x	4	γ	+	+	+	+	+	+	+	+	+	−	+
c	NCTC 10449 GS5 MPK1 UTB2, FW 293 OMZ 70	6	γ	−	+	+	+	+	+	+	+	+	−	
	Ingbritt	1	α	−	+	+	+	+	+	+	+	+	−	
	40 SE, 43 caries, 40 plaques	123	γ/β	−	+	+	+	+	+	+	+	+	−	−
	2 SE, 1 caries, 2 plaques	5	γ/β	−	−	+	+	+	+	+	+	+	−	−
	3 SE, 2 caries, 1 plaque	6	γ/β	−	+	+	+	+	−	+	+	+	−	−
	2 SE, 1 plaque	3	γ	−	+	−	×	+	+	+	+	+	−	−
	2 SE, *1* caries, *1* plaque	4	γ/β	−	+	−	×	+	−	+	+	+	−	−
	1 SE, 1 plaque	2	γ	−	+	+	+	+	+	−	+	+	−	
e	*UTB1 B 2, LM 7*	3	γ	−	+	−	−	+	+	+	+	+	−	−
	2 SE, 4 caries, 2 plaques	8	γ/β	−	+	+	+	+	+	+	+	+	−	
	2 caries	2	γ	−	−	+	+	+	+	+	+	+	−	−
	1 SE	1	γ	−	+		×	+	+	+	+	+	−	−
f	OMZ 175	1	γ	−	+	+	+	+	+	+	+	+	−	
	1 SE, 1 caries, 4 plaques	6	γ/β	−	+	+	+	+	+	+	+	+	−	−
	3 plaques	3	γ/β	×	+	+	+	+	+	+	+	+	−	
d/g	K 1 OMZ 65 RIV 10987 TH 1 C 7	5	α	−	+	−	−	−	×	+	+	+	+	
	B 13 279 144b	3	α	−	+	−	−	−	−	+	+	−	+	
	OMZ 176 SL–1	2	α	−	+	−	−	−	−	+	+	×	+	
	OIHI RIV 10985 RIV 10986	3	α	−	+	−	−	+	+	+	+	−	+	
	TH 21	1	α	−	+	×	+	−	−	+	+	−	+	−
	TH 58, TH 62	2	α	−	+	−	−	−	−	+	−†	×	+	−
	TH 7	1	α	−	−	−	−	−		+	+	−	+	
	TH 40	1	α	−	+	−	−	+			+	+	+	−
ϒ	5 caries, 1 plaque	6	γ/β	−	+	+	+	+	+	+	+	+	−	−
	1 caries	1	γ	×	+	+	+	+	+	+	+	+	−	−
	TH 66	1	α	−	+	−	−	−	−	+	+	−	+	

ϒ = not typeable.
× = delayed positive.
* = NIDR 3720 is reported in other studies (14) to split these substances.
† = negative even if Tween 80 was added to the medium in a concentration of 0.05 per cent.

tive, gram-positive cocci which occurred in short or medium chains. The final pH was 4.1–4.3. All strains failed to grow in the presence of 6.5 per cent sodium chloride, 0.1 per cent methylene blue, and 0.04 per cent tellurite and failed to ferment or hydrolyse arabinose, melezitose, glycerol, glycogen, starch and sodium hippurate. Slime formation and

TABLE 7 *Summary of Table 6*

Serotype	Number	Haemolysis	Arginine	Esculin	Salicin	Raffinose	Mannitol	Sorbitol	Lactose	Dissociation	Biotype
	6	γ/α	0	+	+	5	+	+	5	0	1
b	4	γ	+	+	+	+	+	+	+	+	2
c	150	γ/β	0	140	+	143	+	+	145	+	1
e	14	γ/β	0	+	+	10	+	+	12	+	1
f	10	γ/β	0	+	+	+	+	+	+	+	1
d, g	18	α	0	3	4	1	16	9	17	0	3
SL-1	1	α	0	0	0	0	+	+	+	0	3
λ	7	γ/β	0	+	+	+	+	+	+	+	1
X	1	α	0	0	0	0	+	0	+	0	3

dihydrolase.
+ all strains split the substance.

TABLE 6 *Differences in Biochemical and Cultural Behaviour*

Serotype	Strains	Number	Haemolysis	Arginine	Lactose	Melibiose	Raffinose	Salicin	Esculin	Inulin	Mannitol	Sorbitol	Peroxide	45 C
a	OMZ 49 OMZ 61 HS–1 HS–6	4	γ	–	+	+	+	+	+	+	+	+		
	AHT	1	α	–	+	–	×	+	+	+	+	+	–	
	NIDR 3720	1	γ	–	–	–	+	+	+	+	+	+	–	–
b	FA–1 BHT OMZ 71a, OMZ 51a	4	γ	+	+	+	+	+	+	+	+	+	–	+
c	NCTC 10449 GS5 MPK1 UTB2, FW 293, OMZ 70	6	γ	–	+	+	+	+	+	+	+	+	–	
	Ingbritt	1	α	–	+	+	+	+	+	+	+	+	–	
	40 SE, 43 caries, 40 plaques	123	γ/β	–	+	+	+	+	+	+	+	+	–	
	2 SE, 1 caries, 2 plaques	5	γ/β	–	–	+	+	+	+	+	+	+	–	
	3 SE 2 caries, 1 plaque	6	γ/β	–	+	+	+	+	–	+	+	+	–	
	2 SE, 1 plaque	3	γ	–	+	–	×	+	+	+	+	+	–	–
	2 SE, 1 caries, 1 plaque	4	γ/β	–	+	–	×	+	–	+	+	+	–	–
	1 SE, 1 plaque	2	γ	–	+	+	+	+	+	–	+	+	–	
e	UTB1 B2, LM 7	3	γ	–	+	–	–	+	+	+	+	+	–	–
	2 SE, 4 caries, 2 plaques	8	γ/β	–	+	+	+	+	+	+	+	+	–	
	2 caries	2	γ	–	–	+	+	+	+	+	+	+	–	–
	1 SE	1	γ	–	+		×	+	+	+	+	+	–	–
f	OMZ 175	1	γ	–	+	+	+	+	+	+	+	+	–	–
	1 SE, 1 caries, 4 plaques	6	γ/β	–	+	+	+	+	+	+	+	+	–	
	3 plaques	3	γ/β	×	+	+	+	+	+	+	+	+	–	–
d/g	K 1 OMZ 65 RIV 10987 TH 1 C 7	5	α	–	+	–	–	–	×	+	+	+	+	
	B 13 279 144b	3	α	–	+	–	–	–	–	+	+	–	+	
	OMZ 176 SL–1	2	α	–	+	–	–	–		+	+	×	+	
	OIHI RIV 10985 RIV 10986	3	α	–	+	–	–	+	+	+	+	–	+	
	TH 21	1	α	–	+	×	+	–	–	+	+	–	+	
	TH 58, TH 62	2	α	–	+	–	–	–	–	+	–†	×	+	–
	TH 7	1	α	–	–	–	–	–		+	+	–	+	–
	TH 40	1	α	–	+	–	–	+	–	–	+	+	+	–
X	5 caries, 1 plaque	6	γ/β	–	+	+	+	+	+	+	+	+	–	–
	1 caries	1	γ	×	+	+	+	+	+	+	+	+	–	–
	TH 66	1	α	–	+	–	–	–	–	+	+	–	+	

X = not typeable.
× = delayed positive.
* = *NIDR 3720 is reported in other studies (14) to split these substances.*
† = negative even if Tween 80 was added to the medium in a concentration of 0.05 per cent.

tive, gram-positive *cocci* which occurred in short or medium chains. The final pH was 4.1–4.3. All strains failed to grow in the presence of 6.5 per cent sodium chloride, 0.1 per cent methylene blue, and 0.04 per cent tellurite and failed to ferment or hydrolyse arabinose, melezitose, glycerol, glycogen, starch and sodium hippurate. Slime formation and

duce greening on horse blood agar One of these, a serotype c strain (Ingbritt) was by *Edwardsson* (14) reported to be indifferent, the other (AHT) belonged to serotype a. All strains of serotype a differed culturally from the other strains of biotype 1 in that they lacked the capacity to adhere to the surface of blood agar (see page 362) In this respect they were similar to strains of biotype 3

DISCUSSION

The present study of a total of 210 strains of *Streptococcus mutans* from a variety of sources (Table 1) confirms and extends the studies by *Bratthall* (2, 3) *de Moor et al.* (24) and *de Stoppelaar* (28)

The five distinct serotypes established by *Bratthall*, i.e. types a, b, c, d and e, were also found by us. As supposed by *Bratthall* (4) it was shown that the type e antigen is not identical with the Lancefield group E antigen. In addition, two new serotypes f and g have been established, and strain SL–1 probably represents yet another distinct serotype.

The outcome of serotyping depends to a great extent on the selection of appropriate sera, i.e. specific and potent sera. Thus, the antigenic relationship of type a (HS–1) type d (B 13) type g (OMZ 65) type e (SE 3) and strain SL–1 was disclosed only by the use of such potent antisera. Other antisera, e.g. prepared against type a (HS–1) were not sufficiently potent to demonstrate the common factors in type a and type d by the precipitin test.

The introduction of a new type g related to type d and type a seems reasonable considering that we regard type a and type d as distinct types, although type a and type d, apparently only share one antigenic factor while at the same time *both* type a and type g *and* type d and type g share two antigenic factors.

The FAT studies performed by *Bratthall* (4) with absorbed sera already indicate that common factors exist between type a (HS–1) and type d (B 13) and the type g proposed by us and presented by his strain OMZ 65 which he considered as a type d strain although aberrant from strain B 13 but in order to clearly distinguish the three types a (HS–1) d (B 13) and g (OMZ 65) it is necessary to have at one's disposal absorbed sera specific for the antigenic factors 2, 3 4 and 5

Strain SL-1 could not in our hands be identified with any of the present types. *Kennedy et al.* (20) found a close relationship between SL–1 and HS–1 by agglutination of cell walls. We found no reaction in the precipitin tests and only a faint fluorescence with the FAT in our antisera to HS–1 with strain SL–1

In the present study the serological analysis of strains isolated in this institute (SS) showed that all 54 strains isolated from blood of patients with subacute endocarditis belonged to one of three serotypes c (50 strains) e (3 strains) and f (one strain) Of the 126 strains isolated from human teeth 92 belonged to type c, 9 to type e, 8 to type f one to type d, 8 to type g, and 8 could not be typed. In 6 out of the 8 cases of carious lesions from which type g was isolated strains of type c were present also.

Thus, strains of type a and type b were not isolated from blood or teeth and strains of type d and type g were isolated from teeth only These results are very similar to those reported by *de Moor et al.* (24) and *de Stoppelaar* (28) Among strains from blood these authors found serological group M I (antiserum to NCTC 10449 = serotype c) and a non-typeable (M O) group. Strains of serological group M II (antiserum to strain K 1 which may react with serotypes a, d, or g) were found in teeth only In contrast to the Danish material, the Dutch material contains many non-typeable strains (24.3 per cent in contrast to 4 4 per cent) Strains belonging to types c, e and f might occur among the Dutch non-typeable strains (*Bratthall* (6) typed three of the Dutch M O strains as type e) This is further supported by the fact that *de Stoppelaar* points out that his non-typeable strains have the colonial appearance of group M I (type c) and he "found them

biochemically closely related to that group" The M II strains might belong to type d but not very likely to type a, since the biochemical data given by *de Stoppelaar* (28) indicate that they behave like the presently proposed biotype 3 strains, all of which belong either to type d or to type g, except strain SL–1 which is supposed to be a distinct serotype.

In order to obtain the highest per cent of positive precipitin reactions from formamide extraction, the test should be carried out with two antigen preparations, one obtained from the acid-ethanol precipitate and one obtained from the acetone precipitate. The antigen from the acetone precipitate is in some cases either weakly precipitated or not precipitated at all by the related antisera, while the antigen from the ethanol precipitate generally is strongly precipitated. This compares well with the findings that group antigen was found in both preparations of group F streptococci (23)

For the isolation of strains from teeth we have in part made use of a selective medium containing sulphathiazole. This medium was not used for the isolation of strains from blood. Since four of the seven strains of type d from teeth turned out to be sensitive to this drug, the material originating from teeth is not reliable with regard to the number of isolations of this type. As to the Dutch material, we do not know whether a selective medium was used for isolation. *Bratthall* (5) who did not use a selective medium, found that *S mutants* of serotype d was the serotype most frequently isolated from human teeth in 15 regions in ten countries. As *Bratthall* used an anti-type d serum absorbed with organisms of type a only his type d might cover type g and SL–1 also. The figures in *Bratthall's* study emphasize, at all events, the surprising fact that strains of type d, type g and type SL–1 have never been isolated from the blood of patients with subacute endocarditis.

The regular tolerance to 40 per cent bile registered in the present study is not in accordance with findings otherwise reported in the literature. *Colman* (10) for instance, found that 6 out of 12 strains of *S mutans* did not grow on 40 per cent bile agar. A recent comparison between growth on our bile medium and growth on a medium similar to *Colman's* (dehydrated ox gall added to nutrient agar) showed that some strains would not grow or grew poorly on the latter medium.

The biochemical homogeneity of S. mutans is very conspicuous. Nevertheless, we feel that the registered differences warrant the proposed subdivision into three biotypes (Table 7) Biotype 1 and biotype 2 are differentiated by means of the arginine dihydrolase test and biotype 3 is recognized because *it is on the whole, biochemically more* inert than the two other types.

Apart from the cultural, genetical and serological characteristics of *S mutans* there are two biochemical properties which are unique for this species when present together and therefore highly important for the differentiation of *S mutans* from other streptococci (31) These properties are the fermentation of mannitol and the formation in sucrose broth of a hard mass of growth adhering to the wall of the tube and due to the production of an extracellular insoluble dextran. Out of the 210 strains studied, only two strains, belonging to biotype 3, failed to ferment mannitol.

Comparison of the serological results with the biochemical and cultural behaviour of strains of *S mutants* disclosed a correlation between serotypes and biotypes. *Coykendall* (11 12) found a correlation of his four genetic groups I IV with *Bratthall's* serotypes c, b d and a, respectively and he also found that biochemical differences supported the division into four genetic groups, and proposed variety names for these groups.

Although we cannot recommend *Coykendall's* formal subdivision of the species, we agree that a subdivision has several practical advantages. By using *Coykendall's* subdivision our results can be summarized in the following way

S mutans var cricetus comprises those strains of biotype 1 that belong to serotype a and to *Coykendall's* genetic group IV They

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THE EFFECT OF DIFFERENT SERUM CONCENTRATIONS OF ANTIMICROBIAL AGENTS ON THE *LACTOBACILLUS CASEI* FOLATE ASSAY

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Seventeen antimicrobial agents were added to serum *in vitro* and tested for their ability to influence the assay of folic acid activity by the *L. casei* method. Eight drugs were found to cause significant inhibition. The lowest serum concentrations needed to reduce the estimated folic acid activity by as much as 25 per cent were 0.5-1.0 μg/ml trimethoprim, just below 2.5 μg/ml carbenicillin, 2.5-5 μg/ml lincomycin or phenoxymethylpenicillin, 5-10 μg/ml erythromycin, 10-25 μg/ml benzylpenicillin, just above 25 μg/ml ampicillin and 25-125 μg/ml chloramphenicol.

The microbiological assay of folic acid activity (FAA) may give false low results if the serum contains antimicrobial agents which depress the growth of the test organism. This has been investigated *in vitro* (1 4 7 9) and to some extent *in vivo* (6, 8). Although the same test organism was used, the results are conflicting.

Besides differences in the assay procedure with regard to heating and final serum dilution, the amount of drug present in serum is an important factor.

The purpose of the present investigation is to report how the FAA assay with *L. casei* was affected by different serum concentrations of antimicrobial drugs *in vitro*.

METHODS

Pure drug powder was dissolved in distilled water. Serial dilutions were made and from each of these 0.2 ml was added to 3.8 ml fresh human serum. The FAA assay of this slightly diluted serum was started within one hour.

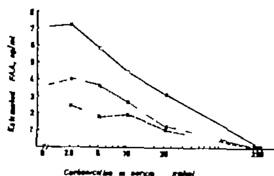
The assay utilizing *L. casei* (ATCC 7469) was performed as described by *Waters & Mollin* (8) with minor modifications. The procedure includes autoclaving at 118° C for 15 min, and the final dilution of serum is 1/100. Incubation lasts until the most concentrated standard solution gives a reading of 0.3 on a Lumson 3 Photometer, usually about 20 hours.

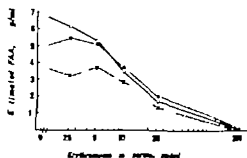
RESULTS

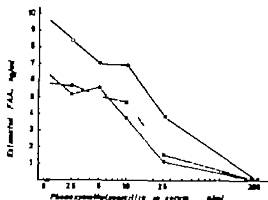
After preliminary screening, 7 drugs were examined at serum concentrations of 250, 25, 10, 5, 2.5 and 0 μg/ml. The only exception was chloramphenicol which was tested at 125 instead of 250 μg/ml because of its low solubility in water. The assays were performed on 3 different days and as a rule with serum from different donors. The results are given in Fig. 1.

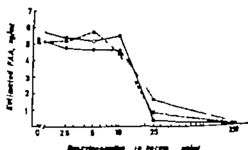
Trimethoprim is commonly used in a fixed combination with sulphamethoxazole, and the relation between their serum concentrations during therapy is about 1/30 (5). The

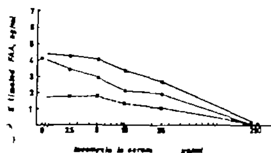
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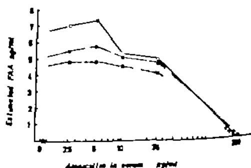












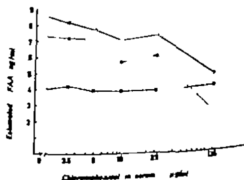


Fig 1 The effect of increasing serum concentrations of 7 antimicrobial agents on the FAA assay. Each agent was tested 3 times.

combination was tested in this relationship at different concentrations with the results shown in Fig 2. When tested separately marked inhibition was caused by trimethoprim, while sulphamethoxazole had no effect.

With increasing drug concentration the reduction of growth always took place gradually. This means that inhibition occurring in a routine assay may be difficult to recognize.

The lowest drug concentration that can cause inhibition is the one of practical importance. The concentrations needed to reduce the estimated FAA by as much as 25 per cent in at least 1 out of the 3 assays were 0.5–1.0 µg/ml trimethoprim (as part of the combination with sulphamethoxazole) just below 2.5 µg/ml carbenicillin, 2.5–5 µg/ml phenoxymethylpenicillin or lincomycin 5–10 µg/ml erythromycin, 10–25 µg/ml benzylpenicillin, just above 25 µg/ml ampicillin and 25–125 µg/ml chloramphenicol.

No significant inhibition was found when cephalotin, tetracycline, oxytetracycline, sulphasodimidin, sulphamethizole or sulphamethoxypyridazine were screened at a serum concentration of 250 µg/ml. Methicillin and streptomycin caused moderate inhibition, but when tested twice at 50 µg/ml, no significant inhibition occurred, i.e. reduction of optical density was less than 15 per cent.

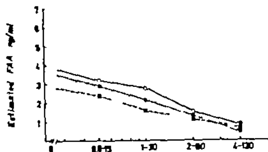


Fig 2 The effect of increasing serum concentrations of trimethoprim + sulphamethoxazole on the FAA assay tested 3 times.

DISCUSSION

Eight antimicrobial drugs have been found to disturb the FAA assay at serum levels ranging from < 1 to > 25 µg/ml. Most of these levels may be exceeded *in vivo*. According to Hellgren (10) the following relationship exists between dose and serum concentration in human adults. If 300 000 U (180 mg) benzylpenicillin is given intramuscularly a peak serum concentration of 8 µg/ml is reached. Similarly 1.0 g sodium ampicillin yields about 10 µg/ml and 1.0 g carbenicillin 10–70 µg/ml. When 0.6 g lincomycin is given intramuscularly every 12 hours, peak serum concentrations of 15–20 µg/ml are obtained. Chloramphenicol reaches a serum level of 20–40 µg/ml if 2 g is given orally. As for erythromycin, a single oral dose of 0.5 g of the stearate gives a peak serum concentration of 2–20 µg/ml and continued medication every 6 hours may yield peaks as high as 50 µg/ml. When phenoxymethylpenicillin is concerned there is also considerable variation between subjects as a single oral dose of 250 mg may after 1 hour give values ranging from < 1 to about 6 µg/ml (2). After an oral dose of 160 mg trimethoprim + 800 mg sulphamethoxazole, a peak trimethoprim concentration of about 1.5 µg/ml is reached after 2–4 hours (5).

It was observed that each drug did not always cause the same degree of inhibition. This may be explained by the fact that the number of viable organisms added to the assay tubes can have varied from day to day. Minor inconsistencies in the autoclaving procedure may also have influenced the final antimicrobial activity.

The relationship between serum drug level and degree of inhibition which was found may explain why earlier investigations have shown, on the one hand, lack of inhibition (9) and on the other hand, almost complete inhibition by all drugs tested (4).

Heating may reduce the activity of penicillin and ampicillin (1) but under the conditions described above inhibition is still of importance. Tetracycline and oxytetracycline, however, disturbing the aseptic addition technique (1) apparently lose all their activity.

Five of the 8 drugs are normally rapidly eliminated from serum *in vivo* Inhibition could therefore be avoided by taking blood in the morning before medication. The absorption of erythromycin may be delayed (3) but even this agent is unlikely to cause inhibition if blood is taken at least 8 hours after the last dose.

The biological half life of trimethoprim is about 8 hours (5) and the half life of lincomycin 5–6 hours (10) During therapy with either of these drugs, the *L. casei* method for serum FAA assay must be expected to give false low results.

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A SIMPLE METHOD FOR DETECTION OF BACTERIURIA WITH AN AUTOMATED CHEMILUMINESCENCE TECHNIQUE

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A sensitive, simple and rapid method for detection of bacteriuria has been developed. By means of a Luminol chemiluminescence method adapted to an Auto Analyzer it has been possible to indicate the presence of low concentrations of bacteria in urine specimens from an unselected clinical material. Less than 1 per cent of the investigated urine samples were false negatives and 7 per cent were false positives according to the Luminol method as compared with viable count. Blood is a disturbing factor. No special treatment of the specimens of urine was needed. The analysing time was only two minutes. Formaldehyde could be added without interfering with the analysis. As the reagents required are inexpensive, and with autoanalyzers available at many hospitals, the Luminol method might be of interest as a mass screening technique for bacteriuria.

The importance of detecting urinary tract infections at an early stage thereby preventing severe chronical cases of renal insufficiency has encouraged efforts to develop uncomplicated but valid screening methods for detection of asymptomatic bacteriuria.

In doing this it is important and necessary to keep apart two problems. One is the detection of bacteria in urine or the changes of the urine initiated by bacteria. The other is the clinical problem of judging whether the patient has or is to have an infection of the urinary tract, especially of the renals.

It is important to observe every clinical or laboratory sign of such a risk, but at the same time the clinical significance of the laboratory findings must not be overestimated.

Thus, it applies to any chemical screening method which is shown to be satisfactorily performed in the laboratory that the interpretation of its clinical significance is another question.

Numerous studies of chemical screening tests for bacteriuria have been published (2, 7 8, 13) Such tests may either measure components or enzymatic activities of the bacteria as such e.g. the Catalase test (1) or may alternatively measure changes in urine composition as the result of bacterial activity e.g. the Nitrite-nitrate test (6 16) the Glucose oxidase test (14 15) and the Tetrazolium reduction test (9)

The principle of the Catalase test is that catalase in bacteria releases bubbles of oxygen if mixed with hydrogen peroxide. As all aerobic cells contain catalase, the specificity of the method is low (numerous false positives) The Nitrite nitrate test (Griess test) is based on the fact that bacteria can reduce nitrate in the urine to nitrite. The specificity of this method is high (few false positives) The sensitivity is low unless precautions with re

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ml to be "significant bacteriuria" Kass emphasizes that many factors must be observed before practical clinical action can be taken based on laboratory values. The clinical situation, the way of sampling the transport of the sample to the laboratory and the analysing method are factors that must be controlled.

METHOD AND MATERIAL

Apparatus

The automated luminol-perborate system was composed of the following instruments from the Auto Analyzer system (Technicon Corp., Ardsley N.Y.) Sampler II Proportioning pump, Fluorometer and recorder A stabilized D-C microvolt amplifier (Leeds and Northrup Company) was connected (Fig. 1) The fluorometer was modified as described by *Ewetz* & *Landin* (3)

Reagents

Luminol, 0.2 g (Aldrich Chemical Co Inc., Milwaukee Wis.) and 3.2 g of anhydrous dextrose were dissolved in 20 ml of 0.2 M N OH. The solution was then diluted to 1000 ml with distilled water The perborate stock solution consisted of a 0.05 M solution of NaBO in distilled water

Luminol Method

The luminol method is based on the fact that luminol (5-amino-2,3-dihydro-1 4-phthalazindione) is influenced by iron containing organic and biological compounds which act catalytically in an oxidation process. The most throughly studied catalyst in this process is ferricyanide (17) Among active biological compounds are cytochromes, peroxidases, catalases, ferritin, haemin and its derivatives (10) and microorganisms. As a rule, they are catalytically active at considerably lower concentrations than ferricyanide. A schematic diagram of the automated luminol-perborate system for detection of microorganisms in water is shown in Fig. 1 The sample is pumped from the sampler through a pump tube mixed with perborate and sodium hydroxide and segmented with air Before entering the flow cell of the fluorometer the sample-solution is mixed with luminol made alkaline with sodium hydroxide. The emitted light is measured in the fluorometer and registered on the recorder The time from the introduction of the sample into the apparatus to registration is only ts minutes.

The intensity of the emitted light is expressed as the scale reading of the recorder in mm. (1 mm corresponds to 0.75 V)

For examination with the chemiluminiscence method the urine specimens were diluted ten times with saline to overcome the slight inhibitory effect of urine on the light emission of luminol, as described by *Weber* (18)

Blood was investigated for its ability to induce chemiluminescence. Whole blood, without anticoagulant, was allowed to stand at 4 C for three hours and then centrifuged at 2000 g for 15 min. Whole blood, red blood cells and serum were then tested with the luminol method. To separate bacteria from red blood cells, whole blood was centrifuged through a membrane filter of 5 μm at 2500 g for 15 min.

Reference Method

The method for determination of the number of bacteria in urine samples routinely used at the Department for Clinical Microbiology at the Karolinska Hospital was used as a reference method to which the results of the luminol test were compared. The method is a semiquantitativ viable count method. Inoculation of plates is made with a calibrated standard loop giving 0.005 ml of urine. Three different media, blood agar base (DIFCO Detroit, Michigan, U.S.A.) endo agar and phenylethyl alcohol agar (BBL, Cockeysville, Maryland, U.S.A.) are used. The petri dishes are incubated at 37 C for 18 h after which the colonies are counted. Findings of less than 50 colonies, corresponding to $<10^4$ bacteria per ml, were registered as negative, 50–500 colonies (10^4–10^5 bacteria/ml) as moderate and >500 colonies ($>10^5$ bacteria/ml) as positive. In 1957 *Kass* (7) stated the limit for significant bacteriuria to be $>10^5$ bacteria/ml urine while 10^4–10^5 bacteria/ml should be regarded

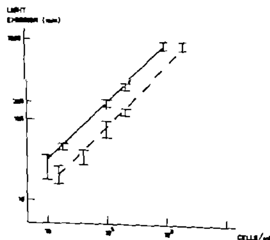


Fig 2 Linear response of Luminol light emission to *Escherichia coli* 053 ——— and *Proteus mirabilis* - - - - in water The vertical bars represent standard error of the mean (n = 4–8)

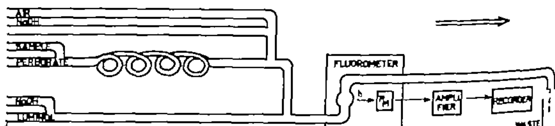
as suspected and $>10^4$ bacteria as negative. Microscopic examination of urine specimens was also made by a conventional method mainly to determine the presence of white and/or red blood cells. Casts, epithelial cells and bacterias were also registered. Proteinuria was tested with Labstix (Ames Company Meda AB, Gothenburg, Sweden)

RESULTS

As has been shown earlier (3 4) bacteria in water are detected by the luminol method in concentrations as low as 10^4/ml (Fig 2) In urine, possible sources of error influencing the Luminol method are iron containing inorganic, organic or biological compounds. In order to differentiate between light emitted by the action of inorganic compounds and that emitted by biological compounds we have utilized the different kinetics of the reactions. The inorganic compounds give a rapid flash of light, whereas biological compounds in contrast give a prolonged relatively stable light emission.

This finding was utilized by prolonging the time between mixing of reagents with the sample and the entrance of the mixture into the flow cell (Fig 3) With a time lapse of 15 sec the light resulting from ferricyanide is no longer detectable, while light resulting from *e.g* cytochromes or bacteria reaches its maximum after 10–15 sec and then slowly decays (5) The time of pre-incubation (60 sec) of the sample together with perborate was of importance, for obtaining the delayed time-course of the haem compounds and bacteria.

To determine the background non specific light emission for negative samples, a separate investigation of urine samples from 25 healthy persons was made. All the samples gave a scale reading of less than 200 mm. 15 gave a scale reading less than 100 mm and 10 gave a scale reading between 100 and 200 As can be seen in Fig 2, 200 mm corresponds to 10^5 *E coli*/ml in water and 2 10^5 *Proteus mirabilis*/ml in water and 50 mm corresponds to respectively 10^4 and 2 10^4 bacteria/ml water



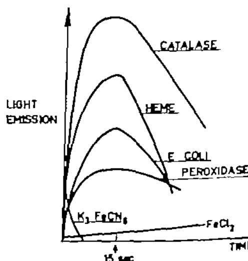


Fig 3 Practical utilization of the kinetic data in the Luminol reaction. The time between mixing of reagents with the sample and the entrance of the mixture into the flow cell was prolonged with 15 sec. Then the *light resulting from* ferricyanide is no longer detectable, while light resulting from e.g bacteria has reached its maximum.

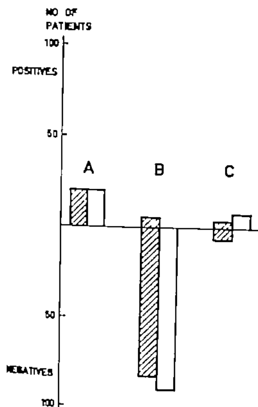


Fig 4 Comparison between results from viable count and chemiluminescence determinations in 119 urine samples.

Reference method (cultivation)
A. Positive >10^5 Bact/ml.
B. Negative <10^4 Bact/ml.
C. Moderate 10^4–10^5 Bact/ml.
Luminol method.

The result of 119 investigated urine samples from an unselected clinical material is shown in (Fig 4) No false negative samples were found according to the limit for significant bacteriuria of 10^5 bacteria/ml. The percentage of false positive samples was 7 per cent. The validity of the empirically determined limit of 200 mm scale deflection was thus confirmed as a tentative limit for practical determination of "significant bacteriuria" Concentrations of bacteria between 10^4 and 10^5 bacteria/ml resulted in scattered values, which might be due to variations in the nonspecific background light emission between 0–200 mm.

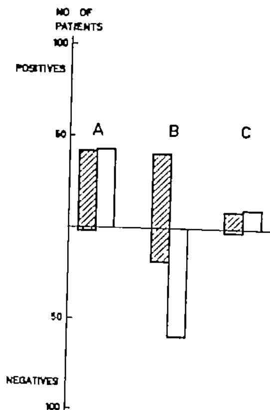


Fig 5 Comparison between results from viable count and chemiluminescence determinations in 115 urine samples from the Department of Obstetrics and Gynecology

Reference method (cultivation)
A. Positive >10^5 Bact/ml.
B. Negative <10^4 Bact/ml
C. Moderate 10^4–10^5 Bact/ml.
Luminol method.

The influence of blood was established. Whole blood could be determined in dilution 1 10^6 in saline, and serum in dilution 1 10^6 with a scale reading corresponding to 5 10^6 *E coli*/ml.

A material of urine samples obtained from the Department of Obstetrics and Gynecology which could be suspected to contain blood, was studied to check whether blood might interfere with the Luminol method otherwise used for practical detection of bacteriuria. As expected, the rate of false positives samples was high, 71 per cent. Among 43 posi-

TABLE 1 *Comparison between Urine Sediment and Chemiluminescence Determinations in 33 Urine Specimens from the Department of Rheumatoid Diseases*

Number of urine-specimens	Urine sediment (per field of view)						Chemiluminescence determination
	Red blood-cells	White blood-cells	Bacteria	Epithelial cells	Hyaline casts	Protein	Scale-reading in mm
29	0–3	0–10	0-single	0-numerous	0-moderate	0-(+)	<200
1	1–3	3–4 pale cells	0	0	single	0	>1000
1	1–3	1–3	positive	0	0	0	300
2	8–1	0–3	0	0	single	0	>2000

tive samples, one was a false negative (Fig 5)

A comparison between the results of urine sediments and the Luminol method was made on 33 adult outpatients from the Department of Rheumatoid Diseases (Table 1) Twenty nine of the urine specimens had a scale reading below 200 mm, i.e the tentatively expected limit of scale deflection. As seen in Table 1 there were no pathological amounts of red blood cells within the range of 11–200 mm. The presence of 5–10 white cells per field of view numerous epithelial cells, moderate numbers of hyaline casts or protein in the urine were not influencing the Luminol reaction. As shown in Table 1 4 out of the 33 urine specimens gave a scale reading >200 mm i.e suspected false positives. The positive Luminol reaction was in one case explained by the presence of bacteria, in two cases by a pathological amount of blood. *In one case pale cells were observed* in the sediment.

Formaldehyde (1 per cent final concentration) was added routinely to the bacteria cultured in broth to stop growth. Experiments were carried out with *formaldehyde added* to urine. No difference was observed whether or not formaldehyde was added. This may be of great importance in the clinical work where time and temperature factors are often overlooked, a possible increase of the bacterial concentration being a consequence.

DISCUSSION

It applies to many chemical tests for bacteriuria that the main disadvantage is the lack of sensitivity leading to false negative results unless precautions are taken, e.g the use of morning urine and restrictions of food intake.

As regards the more sensitive tests, e.g the glucose oxidase and catalase tests, *false negative* results may be caused by metabolic ab*errations in the* patient and kind of infecting organism, respectively In the present investigation, less than 1 per cent of the urine samples gave false negative results. The *proportion* of urine samples giving false positive re*sults is only 7 per cent* in the randomly selected clinical material. As expected, the proportion of false positive results in the potentially blood containing material is very high. In this case, where blood was observed in a great *number of the urine samples.* It was as high as 71 per cent. As shown in this paper the presence of blood has a disturbing influence in dilutions 1 10⁶ Experiments were made to find out whether red blood cells could be *separated by centrifugation* from bacteria, but it turned out that blood serum was detectable in dilution 1 10 probably due to transferrin. The conclusion is that blood in *urine* is a disturbing factor that cannot be overlooked.

This is to some extent supported by the results of the comparison between urine sedi-

ments and the Luminol method, where other cells than red blood cells do not seem to affect the chemiluminescence determination. But it cannot be excluded that the variation of the nonspecific background light emission in urine using the Luminol method, 10–200 mm, can be caused by a varying occurrence of different cells in the urine. As iron porphyrine compounds in both viable and non viable bacteria cause the light inducing ability in the luminol reaction and as these compounds are also present in kidney cells, erythrocytes and leucocytes, the test cannot differentiate between infection and other inflammatory processes in the urinary tract.

Since urine sampled in an adequate way should not contain blood, this lack of differentiating ability between bacteria and blood may constitute no serious limitation to the applicability of the method. In some circumstances it might even be advantageous to be able to indicate in a non specific manner various pathological conditions in the urinary tract, since such observations must always lead to a more detailed examination by more specific methods. The luminol method has been shown to combine a high rate of positively identified bacteriurias (>99 per cent) due to its high sensitivity with an acceptably low rate (7 per cent) of false positives in an unselected clinical material. Thus the luminol method appears to merit further evaluation as a mass screening technique for bacteriuria.

We want to express our thanks to Professor *Hans Ericsson* for his valuable criticism and advice and his generous support. W also want to express our thanks to Drs. *Johan Landin* and *And rs Thore* for their kind support and continued interest, and to Mrs. *Pia Bergström* for skilful technical assistance.

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This is to some extent supported by the results of the comparison between urine sedi-

The strains V1 and V5 are representatives of swine strains, a few of which contained third precipitinogen, poly V The strains were received from Dr *V Hájek* & Dr *E. Marššlek*, Olomouc, Czechoslovakia. For the biochemical and serologic characterization of the strains, see (14)

Isolation of Polysaccharides

Polysaccharides were extracted from whole bacteria with a 1/15 M phosphate buffer pH 6.5 at 37° C and purified on DEAE-cellulose and Sephadex G-75 columns (2.6 × 95 cm) The procedures were mainly as described in (7) except that the last fractionation on Dowex 1 was replaced by gel filtration on Sephadex G-75 columns using an 0.1 M Tris-HCl buffer pH 8.0, with 0.5 M KCl No contamination with nucleic acids or protein was detected spectrophotometrically Polyacrylamide gel electrophoresis with sodium dodecyl sulphate (22) indicated only traces of protein-like material.

Analytical Methods

Acid hydrolysis Samples (5 mg) of polysaccharide were hydrolysed with 2 N HCl for 3 h at 100 C and 6 N HCl for 22 h at 105 C in culture tubes equipped with teflon-coated screw caps. The tubes were flushed with nitrogen before the hydrolysis The HCl was removed by evaporation to dryness *in vacuo* The residues were washed in water and subjected to chromatographic analyses.

Chromatography was carried out on precoated thin-layer chromatography plates (cellulose powder Merck) and Whatman no. 1 paper using five different solvents

A. Butanol Pyridine H_2O–(6/4/3 v/v)
B Ethylacetate Pyridine H_2O–(66/26/8 v/v)
C. Butanol Acetic acid H_2O–(4/1/1 v/v)
D Propanol NH_3 H_2O–(6/3/1 v/v)
E. Butanol:Ethylalcohol:H_2O NH_3–(40/11/49/1 v/v)

Reducing sugars, amino sugars and sugar alcohols were localized with alkaline silver nitrate reagent (21) Reducing sugars were also detected with aniline-phthalate spray (16) Ninhydrin was used for the detection of amino acids (20)

Quantitative determination of neutral carbohydrates and sugar alcohols as trifluoroacetylated derivatives of the corresponding alditols was carried out on a model 900 Perkin Elmer gas chromatograph with glass columns (180 × 0.175 cm) packed with 3 per cent OV 225 (Supelco, USA) coated on Chromosorb W (BDH England) as described by *Imanari et al.* (9) and by *Endresen* (in press) Quantitative analysis of amino acids was also performed by gas liquid chromatography (GLC) (19) Hexosamines were determined by a modified Morgan-Elson method (10) and phosphorus as described in (23)

Alkali hydrolysis Ten mg of each polysaccharide (except V5) was hydrolysed in sealed Pyrex tubes with 0.5 ml 1 N NaOH for 3 h at 100° C. The hydrolysates were passed through 1 × 4 cm Dowex-2 (AG2– ×10 acetate form, Bio-Rad, USA) columns. The columns were eluted with 4 × 2 ml of distilled water and the eluates neutralized with 2–3 ml Amberlite IR 120 hydrogen form (Koch-Light, England) and then lyophilized (*fraction A*)

The columns were then eluted with 2 ml of 3 N HCl, followed by 2 × 2 ml of distilled water The eluates were evaporated *in vacuo* over KOH pellets (*fraction B*)

Samples were chromatographed on Whatman no. 1 paper using solvent D Products were detected by the benzidine-sodium metaperiodate spray (20) and molybdate reagents for phosphoric esters (6)

About one half of each fraction B was treated with 0.2 mg of alkaline phosphatase (Type II, Sigma, USA) in 0.01 M $(NH_4)_2CO_3$, pH 9.5 for 1 h at 37 C (3) The enzyme was precipitated by 2 N HCl and removed by centrifugation. The acid supernatants were heated for 3 h at 100° C in sealed tubes, and evaporated to dryness. The resultant residues were examined by chromatography

Reaction with Concanavalin A

Concanavalin A isolated from jack-bean meal (Sigma) by the method described in (1) was used in a concentration of 25 mg per ml of phosphate-buffered saline, pH 7.2 (PBS) The polysaccharides were used in concentrations of 1 mg per ml PBS. Poly Aβ (7) and poly Bα (12) were included as a negative and a positive control. The reactions were performed in 1 per cent agar

Serological Methods

Immune sera were produced against formalin-killed bacteria by intravenous injections (13) into New Zealand white rabbits of the Institute's breed. Double diffusion in agar was performed as described in (8) Reference systems for poly Aβ (7) poly Bα (12) and poly C (12) were included. Im-

TABLE 1 *Precipitation Reactions in Agar*

Polysaccharide (1 mg/ml)	Rabbit antisera against strains: H1	P1	P4	Z14	V1	V5
H1	+	—	—	—	—	—
P1	—	(+)	+	+	—	—
P4	—	±	+	+	—	—
Z14	—	±	+	+	—	—
V1	—	—	—	—	+	+
V5	—	—	—	—	+	+

munoelectrophoresis was performed using an LKB apparatus as described by the manufacturer (LKB Produkter)

RESULTS

Purified polysaccharides The results of agar gel precipitation are presented in Table 1 All the polysaccharides gave single precipitation lines. Three separate lines were detected. One was produced by H1 a second line was shared by Z14 P1 and P4 and a third line by V1 and V5 Antiserum V1 was weak and gave indistinct lines. Poly H1 gave complete cross-reaction with poly C (12) and a reaction of partial identity with poly Aβ The spur formation against the poly Aβ line reported with V1 bacteria (14) could not be reproduced with poly V1

Concanavalin A reacted in double diffusion with all the polysaccharides except poly H1 On immunoelectrophoresis in agar the polysacharides moved like poly A, being acid polysaccharides.

Acid hydrolysates Analysis of the acid hydrolysates of the polysaccharides by paper and thin layer chromatography showed that glycerol was present in all samples whereas ribitol (or anhydroribitol) could not be demonstrated. Further muramic acid and the typical murein amino acids were found, indicating that the polysaccharide was linked to murein fragments. Poly H1 contained glucosamine, poly Z14 P1 and P4 glucosamine and glucose, and poly V1 and V5 glucosamine, glucose galactose and mannose (Table 2) Acid hydrolysates of the polysaccharides also contained glycerol phosphates. This was shown by treatment with alkaline phosphatase, after which free glycerol and phosphate could be demonstrated.

The results of the quantitative determinations by GLC are shown in Table 2.

Alkali hydrolysates Fraction A, obtained after elution from the Dowex 2 column, contained free glycerol and glycerol sugar compounds without phosphate. These were eluted from the paper after preparative chromatography in solvent D and then hydrolyzed for 3 h in 2 N HCl. Chromatography of the residues in solvent D showed that glycerol and glucose were present in poly Z14 P1 and P4 whereas poly V1 in addition contained galactose and mannose. Fraction A of poly H1 in addition to glycerol, contained only trace amounts of partially degraded material

Chromatography in solvent D of fraction B of the poly H1 hydrolysate revealed two spots by the molybdate reagents. Enzymatic dephosphorylation after elution of corresponding bands in preparative chromatography on

TABLE 2. *Chemical Analyses*

Polysaccharide	H1	Z14	P1	P4	V1	V5
			µmole/mg*			
Glycerol	1.22	0.92	0.71	0.79	0.85	0.62
Ribitol	—	—	—	—	—	—
Glucose	—	0.53	0.31	0.59	0.21	0.17
Mannose	—	—	—	—	+	+
Galactose	—	—	—	—	+	+
Glucosamine	2.86	0.78	0.33	0.49	0 60	0.83
Muramic acid	+	+	+	+	+	+
Phosphorus	2.53	1.59	1 63	1.56	1.50	1.65
Alanine	0.18	0.32	0 41	0 48	0.68	0 61
Glycine	0.30	0.55	0.68	0.84	1.05	0.98
Lysine	0.10	0.17	0.22	0.28	0.33	0.29
Glutamic acid	0.09	0.15	0.18	0.26	0.31	0 28
Unidentified sugar	—	—	—	—	+	+

*Mean of three analyses.

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Glucose	—	0.53	0.31	0.59	0.21	0.17
Mannose	—	—	—	—	+	+
Galactose	—	—	—	—	+	+
Glucosamine	2.86	0.78	0.33	0.49	0.60	0 83
Muramic acid	+	+	+	+	+	+
Phosphorus	2.53	1.59	1 65	1.56	1.50	1.65
Alanine	0.18	0.32	0.41	0 48	0.68	0 61
Glycine	0.30	0.55	0.68	0.84	1.05	0.98
Lysine	0 10	0.17	0.22	0.28	0.53	0.29
Glutamic acid	0.09	0 15	0.18	0.26	0.31	0 28
Unidentified sugar	—	—	—	—	+	+

Mean of three analyses.

Whatman no. 3 MM paper released free in organic phosphate from both. After hydrolysis of the sample (fraction B) for 3 h in 2 N HCl, glycerol and glucosamine were detected. Four organic phosphate fractions were detected after paper chromatography of the alkali hydrolysates of poly Z14 P4 and V1. Due to lack of material these organic phosphates were not subjected to further analyses.

DISCUSSION

Both serologically and chemically the polysaccharides isolated from strains Z14 P1 and P4 seemed to be identical. The same seemed to be true for the polysaccharides from strains V1 and V3. Thus, in accordance with the previous serologic examination of whole bacteria (14 15) three different polysaccharides, viz. poly H, poly P and poly V were isolated from these animal strains of *S aureus*. All the three polysaccharides contained glycerol, phosphorus, sugars and amino sugars in addition to muramic acid and the typical staphylococcus murein amino acids. Ribitol or anhydroribitol were not detected. The ratio of glycerol to phosphorus ranged from 0.38 to 0.57 whereas typical polyglycerophosphates have a ratio near 1 0. This discrepancy can be explained by the presence of glycerol phosphate complexes after acid hydrolysis, resulting in a reduced amount of free glycerol.

Of the three polysaccharides poly H has been best characterized. The alkali hydrolysate contained glucosaminylglycerol and glucosaminylglycerolphosphate. Poly H thus appears to be a glycerol teichoic acid. Glucosamine was present in unusually large amounts. Poly H did not react with concanavalin A. This result, together with the serologic cross-reaction of poly H with the poly Aβ system, indicates that the glucosamine is in the β-position and that β-glucosamine is the common serologic determinant of poly H and poly Aβ containing different polyols.

The nature of the two other polysaccharides is more uncertain. Glucosylglycerol compounds were detected in the alkali hydrolysate of poly P and glycerol combined with glucose, galactose and mannose in poly V. Four organic phosphate compounds were detected in fractions B of the alkali hydrolysates, but due to lack of material the composition was not established. Nevertheless, poly P and Poly V probably are glycerol teichoic acids of a more complex and unusual type. Both polysaccharides reacted with concanavalin A in agar indicating the presence of terminal α-glycosidic residues (4 17). As poly P contained relatively large quantities of glucose, α-glucose may be the antigenic determinant. If this were true a cross-reaction with poly Bα of *S epidermidis* (12) would be expected. Such a reaction was, however not observed.

Poly H seems to be the characteristic teichoic acid of pigeon and mink *S aureus* strains and poly P was present in the majority of dog strains (14 15). Thus these substances are of interest for classification and taxonomy. Further chemical studies are in progress to achieve a more detailed knowledge of poly P and Poly V.

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DEMONSTRATION OF CARCINOEMBRYONIC ANTIGENS (CEA), NONSPECIFIC CROSS-REACTING ANTIGENS (NCA) AND AN ASSOCIATED ALPHA PROTEIN IN NORMAL HUMAN TISSUES AND FLUIDS BY IMMUNODIFFUSION TECHNIQUES

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Perchloric acid extracts (PCA) of cancer tissues, normal and inflammatory tissues, normal sera, urines and salivas were studied to detect carcinoembryonic antigens (CEA) and cross-reacting a tigens The extracts were tested in high concentration by immunodiffusion met hods in agarose gel. Substances which showed complete identity with CEA were found in pparently normal tissue, saliva and urine and in inflammatory tissue as well as in primary lung cancer In addition, material which cross-reacted with CEA was found in all the samples tested in adequate concentrations, including serum, saliva and urine. Both CEA and the non-specific cross-reacting antigens (NCA) were found in samples of purified blood group substance A and B and in saliva from secretor and non-secretor An α-protein was found in PCA extracts of normal and cancer tissue normal serum and some urines. Some preliminary observations are reported on the α-protein which seems to share antigenic determinants with the β_E-protein possessing NCA determinants.

In a previous study two proteins with β-mobility found in perchloric acid extracts (PCA) of colonic adenocarcinomas were described (21) One of the proteins, called β_I, had antigenic determinants identical to GOLD's carcinoembryonic antigens (CEA) The other protein, called β_E, had no CEA determinants, but evidence was presented that the β_E-protein shared some antigenic determinants with the β_I protein. Similar observations have been reported by others, and antigenic sites common to β_I- and β_E protein have now been called non-specific cross-reacting antigens or NCA (7 16)

The β_E-protein was found in perchloric acid extract of normal colonic mucosa (21) Later another protein with α mobility was observed in the same extract and some evidence was presented that the α-protein shared antigenic determinants with β_E-protein (22)

Antibodies against common determinants, like NCA, may cross-react with β_I and β_E proteins and interfere with the specificity of the CEA test in immunodiffusion studies and even more in highly sensitive methods, like radio-immuno-assays Recent studies by radio-immuno-assay indicate that CEA or CEA-like

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materials are present in tumours outside the gastro-intestinal tract in non-cancerous tissues, in sera from patients with liver cirrhosis and in some normal sera (4 5 9 10 11 12, 14 17 20) Positive CEA reaction has also been demonstrated in urine from patients with urothelial carcinoma (3) and association with blood group substances has been suggested (19) A possible explanation of these findings could be that NCA or other *antigens found in normal tissue and fluid* were involved in the reactions.

This study which has been preliminarily reported elsewhere (22) was done to see if CEA as well as NCA and antigens associated with the α-protein could be detected and identified in normal human tissues and fluids. Some pathological human materials, such as mucosa from ulcerative colitis, hydatidiform mole, pulmonary carcinoma and urine from patients with genital carcinoma were included. To be able to distinguish between CEA and cross-reacting antigens in the most proper way immunodiffusion techniques were used, which are less sensitive than more sophisticated methods, but they have the advantage that the specificity as well as identity and cross-reactions can be more easily recognized.

MATERIALS AND METHODS

Perchloric acid extracts (PCA) were prepared as previously described (21) from the following materials. Nine apparently normal colonic mucosas, *one of which was surgically removed more than 10* cm from a colonic tumour the others were taken at autopsy from patients who had died of diseases other than cancer or from patients with cancer in other organs. Colonic mucosa, with pathological changes, from four patients operated for ulcerative colitis were included, as well as four liver tissues which were normal by macroscopical examination and taken at autopsy from patients who had died of non-cancerous diseases. Included were also four normal human sera from persons of blood group A, one sample of meconium, one sample of a hydatidiform mole two pulmonary tumours and eight samples of urine. Two of the samples of urine were from healthy persons, and six were from patients with carcinoma uteri or cervix uteri receiving X-ray therapy. Pyuria was observed in all the patients, some with and others without bacteriuria.

The *surgical specimen were collected immediately* after removal, while autopsy materials were collected from 25 to 32 hours post mortem. All the samples, including urine meconium and the hydatidiform mole were frozen and stored at 25 C untill extracts were prepared and freeze-dried The tests were performed at the highest possible protein concentration, ranging from 40–165 mg/ml, as measured by the Folin method.

In addition saliva from four healthy persons of blood group A was tested two of the samples were from non-secretors and two were from secretors. The saliva was boiled for 10 min immediately after collection to inactivate enzymes Cell fragment and other solid materials were then removed from the saliva by centrifugation at 10,000 g for 15 min.

Purified blood group substances of A, B, H and Le^a specificity prepared from human ovarial cysts, were kindly provided by Drs. *Marge* & Heiben. Comparison was made with blood group substance prepared from animal materials, e.g. porcine and equine AB specific substance from Merck Sharp & Dohme. The salivas and blood group substances were not PCA extracted but freeze-dried and tested as a viscous solution at the highest possible concentration.

Immunoelectrophoresis and double immunodiffusion tests were performed as described, (21) Weak precipitin lines were made better visible, if necessary by incubation of the immunodiffusion plates in 4 per cent tannic acid for 30 min after the plates were washed in phosphate buffered saline for 48 h.

Perchloric acid extract of colonic adenocarcinoma was run on a Sepharose B4 column, fractions containing the β_1 and β_2-proteins were further separated on a Sephadex G-200 column using a phosphate buffer of 0.1 M, pH 7.2 7.5 in 0.03 [illegible] NaCl. The β_1-protein (CEA) was eluted in the first peak and the β_2-protein (NCA) in the second *peak, which in addition* also contained the [illegible] eluted α-protein. Good separation of the two peaks was obtained. The Sephadex G-200 fractions were tested in protein concentrations of 2–5 mg/ml.

Precipitin inhibition tests were done as described (21) The secretor status was determined by conventional methods (2)

Antisera

Four antisera were used. Antisera nos. 57 and 14 which have been described previously (21) gave identical reactions They were produced in rabbits immunized with PCA extract of colonic adenocarcinoma and primarily absorbed by normal human serum and by PCA extract of normal colonic mucosa or liver tissue as described (21) Fur-

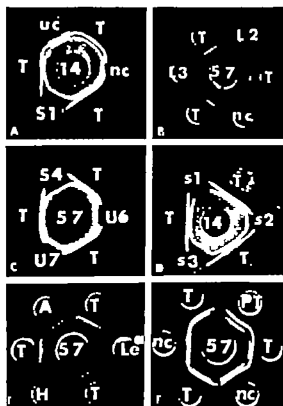


Fig. 2. Demonstration of CEA and NCA by immunodiffusion in agarose gel.

Central wells: Antisera nos. 14 or 57 primarily absorbed (21).

Peripheral wells: The material tested. The wells T are included as controls and contain extract of colonic carcinoma.

The β_I lines (CEA) are closest to the peripheral wells and the β_E lines (NCA) closest to the central wells (21).

A) CEA and NCA are demonstrated in normal colonic mucosa nc, and in mucosa from ulcerative colitis, uc. NCA, but no CEA, is seen in the normal serum sample 81.

B) Weak NCA is demonstrated in two normal liver extracts L2 and L3. (The reaction is not well reproduced on the photograph.) A strong CEA reaction is seen in L3, no CEA reaction in L2. (Both extracts are tested at protein concentrations of 165 mg/ml.)

C) Strong NCA reaction is seen in the two urines, U6 and U7, and in the normal serum sample 84. No CEA reaction is seen in the serum sample but trace reactions of CEA are found in the urines. U6 is urine from a patient with carcinoma cervix uteri. U7 is a normal urine.

D) NCA is demonstrated in salivas, s1, s2, s3. A trace reaction of CEA is seen in the saliva s2. This saliva is from a non-secretor. (The trace reaction is not well reproduced on the photograph.)

E) CEA reaction against blood group substance A is seen (well A). No reaction against H and Le^a substance is observed (well H and Le^a). On the original plate, trace reactions of NCA were also detected in A, H and Le^a substance.

F) A strong CEA as well as NCA reaction is seen in one of the patients with pulmonary tumour PT. (Different samples from normal colonic mucosa are included in wells nc. Against none of the extracts a weak precipitin line unrelated to β_I or β_E is seen.)

from patients of blood group O as well as group A. In saliva, traces of CEA were found in two cases, while two other cases were CEA negative. One of the CEA positive samples was from a secretor, the other from a non-secretor; both were of blood group A. Traces of CEA were found in three out of eight urines. Two of the urines were from cancer patients with pyuria, one with bacteriuria, the other without. One was from an apparently healthy person.

Some of the extracts of normal liver and colonic mucosa were CEA negative, while others were strongly positive. This variation could not be explained by the variation in protein concentration in which they were tested. One of the liver extracts (Fig. 2B, L2) was CEA negative when tested at a protein concentration of 165 mg/ml, though another liver extract (L3) was CEA positive down to a concentration of 30 mg/ml.

NCA were found in all the human materials tested in appropriate concentrations, including samples of purified A, B, H and Le^a substances, though weak reactions were obtained in the purified blood group material. Two urines which were tested in protein concentrations between 5–10 mg/ml were NCA negative and negative reactions were also seen in the animal blood group substance.

CEA like material was demonstrated in the meconium extract, where reactions of partial identity were seen with the β_I as well as the β_E-line, indicating that meconium was

TABLE 1 *Demonstration of CEA and NCA by Immunodiffusion Studies in Agarose Gel*

Materials	CEA pos/tested	NCA pos/tested
Colonic mucosa, normal	7/9	9/9
Colonic mucosa, ulc. colitis	4/4	4/4
Liver tissue normal	1/4	4/4
Primary pulmonary cancer	1/2	2/2
Human serum, normal	0/4	4/4
Meconium	1/1	1/1
Urine, normal	1/2*	2/2
Urine, cancer patients	2/6	4/6†
Hydatidiform mole	1/1	1/1
Saliva, normal, secretor/non-secretor	2/4	4/4
Blood group substance		
Human, A specificity	1/1	1/1
Human, B specificity	1/1	1/1
Human, H specificity	0/1	1/1
Human, Le^a specificity	0/1	1/1*
Porcine and equine, AB specificity	0/1	0/1

The tests were performed in perchloric acid extracts in all cases except in samples of saliva and blood group substances, which were tested after concentration by freeze-drying. With two exceptions† the samples were tested at concentrations between 40–165 mg/ml protein, which was the highest possible concentration suitable for testing.

*Trace reaction

†The 2 neg. tested in conc. <10 mg/ml.



Fig. 3. Inhibition of anti-CEA and anti-NCA by blood group substance B.

Well A. Blood group A substance.

Well B. Blood group B substance.

Well T. Perchloric acid extract of colonic carcinoma.

W II 57. Primarily absorbed antiserum no. 57

W II 57a. Primarily absorbed antiserum no. 57 inhibited by blood group B substance, (0.7 ml conc. sol. per ml antiserum)

The strong CEA- and weak NCA-lines are inhibited by the B substance. The weak reactions, still seen, against well B and T became completely negative after inhibition by a double quantity of B substance. Addition of saline in equal amounts did not effect the reaction.

deficient in CEA determinants compared with the tumour extract. These reactions were not studied in further detail.

The identity of CEA which was demonstrated in normal tissue and in purified blood group substance was further studied by inhibition experiments. The CEA precipitin line against colonic tumour extract was completely inhibited by 37 mg of normal liver extract (no. L3) added to one ml of antiserum no. 57. Inhibition was also seen with the other extracts of normal tissue, though complete inhibition was difficult to achieve with some of the normal extracts, which had low concentration of CEA active material. Similar results were obtained by inhibition with purified blood group substance as seen in Fig. 3 where inhibition with B substance completely removed the CEA reaction against colonic tumour extract.

The occurrence of the α-protein was studied in a series of experiments by immunoelectrophoresis using the antisera nos. 25 and

activity in different samples, may explain why many previous studies have concluded that CEA were not present in normal tissue. To explain the variations of CEA in normal tissues, changes due to age may be considered. The normal tissues in our study were from patients in the 60–70 age group. The strong CEA reactions found in some of the apparently normal tissue extracts may also be due to tissue changes caused by the actual disease or to possible post mortem changes. Further studies are needed to answer these questions.

We did not detect CEA in normal serum where only NCA could be demonstrated by immunodiffusion techniques. Similar observations have been made by others (1). The negative CEA reaction may be due to the low sensitivity of the immunodiffusion method compared with radio-immuno-assay techniques, by which CEA-like materials have been demonstrated.

CEA or CEA like materials have been demonstrated in the urine from many patients with transitional cell carcinomas of the bladder (3). The strong NCA reactions, which we found in all our samples of urine tested in adequate concentration, show that it is possible to detect CEA cross-reacting materials, instead of CEA, also by examination of urine. On the other hand, using immunodiffusion methods, weak CEA activity was demonstrated in one of our samples of normal urine and in two urines from cancer patients with pyuria. The number of urines studied was too small to give further information about the cause of these findings, e.g. influence of cellular fragments, bacteria or other urinary components. The low CEA concentration in urine also made the immunodiffusion method less suitable for such studies. Other studies seem to indicate that the positive CEA reaction is not due to the bacterial contamination itself (3).

Weak CEA activity in saliva has recently also been reported by others (13). Gel-filtration studies and immunoelectrophoresis indicate that CEA in saliva and in colonic tumours may be identical (13). Demonstration of CEA in saliva, independent of the secretor status, may suggest that CEA are associated with molecules that are different from those possessing active blood group specificity. This would imply that the strong CEA activity found in the purified A and B blood group substance tested by us was due to heterogeneity or impurity in this material. In fact, we were able to show that the purified blood group substance gave two precipitin lines, one identical to β_I, the other identical to the β_E-line. These substances were therefore not homogeneous, and impurities of CEA or β_I-protein could be present as well as impurities of NCA or β_E-protein.

We found negative CEA reaction in H and Le^a substance. Negative CEA reactions in some samples of A and B substance have also been reported (18). The findings of CEA in some preparations of blood group substances, but not in others, may further speak in favour of impurities rather than cross-reaction between CEA and blood group substance due to structural similarities. Such common structures, however, have been indicated by inhibition studies with selected oligosaccharides possessing blood group activity (19). The activity against A and B specific sites was removed from our antisera by absorption with human red cells, and cross-reaction with A or B immunospecific sites could therefore be excluded.

Further tests with human anti A and anti B sera could possibly solve this problem. However, immunodiffusion experiments with a number of hyperimmune anti A and anti-B sera did not give precipitation reactions sufficiently strong for such studies.

By antisera nos. 25 and 26 we were able to demonstrate an α-protein which has not yet been identified. The α-protein was not found to be identical to the α-protein which possesses the third main antigen (MTA) described by *v. Kleist & Burtin* (6, 8). The indicated cross-reaction between our α- and β_E-protein shows that this protein may have some association with the CEA NCA system and may interfere with the specificity of the NCA-test, possibly also with the CEA test.

The α protein was found in PCA extracts of normal tissues and normal sera as well as in extracts of cancer tissues and urines. The concentration of this protein was rather low though higher than the β_E-concentration and high enough to be detected in PCA extracts by immunoelectrophoresis There was a striking difference in the reaction pattern of the α-protein in different samples, with spurformation between α- and β_E-protein in some samples and reaction of apparently complete identity in others. This observation may suggest that variants of this protein exist. Further characterization and identification of the α-protein and its relations to other proteins in the PCA extract of normal and cancer tissues is in progress.

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microscopy that stripping off the HBAg positive coat of the Dane particle by means of a detergent (Tween 80) gives rise to a 27nm particle resembling a picorna virus. Such particles are aggregated by sera from post hepatitis B patients although these sera do not contain HBAb (1 14) Since this quality seems to be present in convalescent sera more constantly than the HBAb, it may be utilized as a new tool by which to establish a diagnosis of virus B hepatitis.

This publication reports the detection by a gel diffusion test of a new antigen-antibody system intimately connected with virus B hepatitis. It also presents indications that the "new" antigenic determinant is located on the 27nm "core" of the Dane particle, and the implications of these findings are discussed.

MATERIAL AND METHODS

Reagents

I Sera. 1 A commercial, human antiserum towards HBAg (Spectra Biologicals, lot.no. 162)

2. A serum negative for HBAg or HBAb which is aggregating the 27nm particles in immuno-electron microscopy This serum was a gift from Dr *June D. Almeida* to whom we are indebted.

3. The serum of a patient (A.T.) who had undergone an HBAg positive hepatitis 6 months prior to collection of the serum. As previously reported, this serum aggregates 27nm particles, and is negative for HBAg and HBAb by counter electrophoresis and closed hexagon immunodiffusion (CHI)

4 The serum of patient (O.A.S.) hospitalized on account of chronic hepatitis. This serum demonstrated considerable amounts of 20nm particles, tubules and Dane particles and in addition some free 27nm particles (Figs. 4a and 4b) This serum as the source of antigen preparations for gel diffusion.

5 A serum from a long-term HBAg carrier (I.A.) This serum contains 20nm particles and tubules but no Dane particles are demonstrable by electron microscopy

6. The serum of an individual (E.K.) with no history of hepatitis This serum is negativ for HBAg and HBAb by the techniques mentioned above and it contains no particles demonstrable by electron microscopy

II Detergent Tween 80 (polyoxyethylene sorbitan mono oleate) "Koch & Light" was employed in these experiments.

III Dextran T 250 Pharmacia was used on account of its beneficial effect on gel diffusion results (2)

Ultracentrifugation and Detergent Treatment

The serum was cleared by centrifugation at 5000 × *g* (7000 rev/min) for 20 minutes in a Sorvall RC2 B centrifuge with rotor SS-34 The supernatant was diluted from 10 ml to 30 ml with phosphate-buffered saline (PBS) and centrifugated for hours at 100000 × *g* (40000 rev/min) in a Christ ultracentrifuge with angle rotor 9720 The pellet was suspended in 30 ml PBS or 5 per cent Tween 80 in PBS and incubated for 30 minutes at room temperature. The suspension was centrifugated for 2 hours at 100000 × *g* The pellet was resuspended in 30 ml PBS and the suspension was centrifugated for 2 hours at 100000 × *g* The pellet was suspended in 0.2 ml PBS and used as antigen in immunodiffusion and immuno-electron microscopy

Preparation for Immuno-electron Microscopy

0 1 ml suspension of detergent treated antigen or 0.5 ml untreated HBAg positive serum was added to 0.1 ml antiserum. The suspension was mixed carefully and incubated overnight at 4 C. After incubation the suspension was diluted to 5 ml with PBS and centrifugated for 1 hour at 20000 × *g* in Sorvall RC2 B centrifuge rotor SS-34. The pellet was resuspended in 5 ml PBS and centrifugated for 1 hour t 20000 × *g* The supernatant was discarded and the pellet was submitted to electron microscopy after staining.

Electron Microscopy

Pellets obtained after preparation for immuno-electron microscopy were suspended in 0.1 ml distilled water One drop of this suspension was mixed with one drop of 3 per cent phosphotungstic acid, adjusted t pH 6 with KOH, and one drop of this mixture was placed on a 200 mesh carbonformvar coated grid. Excess fluid was removed with filter paper and the grid was examined in a JEM electron microscope at a magnification of 60000 ×

Gel Diffusion

Gel diffusion tests were performed by closed hexagon immuno diffusion (CHI) a sensitive modification of the Ouchterlony technique The method has been described recently (13) Briefly the immuno diffusion takes place in a closed hexagonal Perspex chamber with angles curved to fit the gel-puncher Agarose (L'industrie biologique Francaise) including 2 per cent Dextran T250 in PBS pH 7 4 was employed for the preparation of gels. Since preliminary experiments had shown a 1 1 per cent gel to giv favourable re-

sults, this concentration was utilised throughout the experiments. The inclusion of Dextran T250 causes sharper precipitation lines (2) The diffusion chambers were incubated at room temperature for 48 hours and then further incubated at 4 C for several days before the final reading was done. The sides of the hexagon are 10 mm, and a 3 mm diameter gel puncher is employed for preparing the wells. Average gel thickness is 1 7 mm, but due to wall effects, the gel will be thicker at the periphery of the hexagon.

Staining of the Gels

The gels are washed in PBS for 24 hours with a magnetic stirrer Frequent changes of saline (4–5 times) are essential. On the next day the gels are treated for 30–60 minutes with a 4 per cent solution of tannic acid in PBS and washed once in distilled water

RESULTS

1 Electron Microscopy

The serum of a patient (O.A.S) with chronic hepatitis was earlier shown to be positive for HBAg in gel precipitation (CHI) counter electrophoresis and CFT (Titre 128) In the electron microscope, this serum demonstrated all the three particle forms connected with a positive HBAg reaction i.e. the spherical 20nm particle, the tubules and the 42nm Dane particles (Fig 1) But in addition, a few approx. 26–28nm particles with "capsomerelike" repeating subunits was observed (Figs. 2 and 3)

If serum O.A.S was treated with Tween 80 and subsequently reacted with posthepatitis sera (Almeida's anticore and A.T) which are negative for HBAb, aggregates of 27nm particles were demonstrable in the electron microscope (Figs. 4a and 4b) Besides, 20nm particles were observed to be randomly distributed together with some short tubules disintegrating in a manner recently reported from this laboratory

2 Closed Hexagon Immunodiffusion (CHI)

To obtain information of the specificity of the precipitation lines observed in closed hexagon immunodiffusion, various experiments including several controls were carried out. One particular experiment was repeated several times before the results were accepted. Some of these experiments are reported here.

Experiment 1

After it was established that detergent treated pellets from serum O.A.S. gave a precipitation line when diffused against post hepatitis sera (Almeida's anticore and A.T) negative for HBAb, we found it most urgent to establish whether Tween 80 alone or in connection with an HBAg negative serum could produce the same effect. For this purpose, a serum (E.K.) negative for any of the three particle forms of HBAg was detergent treated in parallel with serum O.A.S., and these sera were tested against post hepatitis serum in CHI. In addition, one of the wells was filled with a 5 per cent Tween 80 solution. The negative serum and the Tween solution produced no precipitation lines

Experiment 2.

In this experiment, a post-hepatitis serum (Almeida's anticore) was placed in the central well, the detergent treated O.A.S. serum being placed in two opposite wells the other peripheral wells contained A serum of a long term HBAg carrier (I.A.) a commercial human HB-antiserum (SaAu) (Spectra Biologicals) an HBAg/Ab negative serum (E.K.) and another post-hepatitis serum (A.T) The precipitation lines observed are illustrated in Fig 5 This experiment revealed that both the post-hepatitis sera negative for HBAb, the commercial human HB-antiserum and the long term HBAb carrier serum possess some common antibody population which reacts with an antigen in the detergent treated serum (O.A.S.) containing Dane particles. This new antigen-antibody system clearly demonstrates non-identity to the common HBAg/Ab system.

Experiment 3.

Since the serum O A S had demonstrated some "naked" virus-like particles of approx 27nm diameter without detergent treatment,

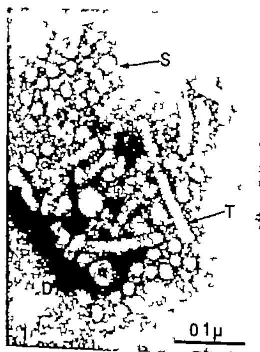


Fig 1 HB-antigen-antibody complex from serum of patient with chronic hepatitis (O.A.S.) reacted with HBAb. Three morphologically different particles are visible. Magnification 180000 ×

All preparations for electron microscopy were negatively stained with 3 per cent phosphotungstic acid pH 6. The following abbreviations are used S = 20nm particles, T = tubular form, D = Dane particle, P = 27nm particle, SaAu = Commercial human antiserum towards HBAg (Spectra Biologicals) AG and AT = Two sera negative for HBAg and HBAb, but aggregating 27nm particles in immuno electron microscopy OAS = Serum containing 20nm particles, tubular forms, Dane particles and free 27nm particles. I.A. = HBAg positive serum containing 20nm particles and tubular forms but no Dane particles demonstrable by electron microscopy E.K. = Serum negative for HBAg and HBAb by counter electrophoresis, closed hexagonal immunodiffusion and electron microscopy



Fig 2 As in Fig. 1 20nm spherical particles and tubular forms and one single free 27nm particle. Magnification 180000 ×



Fig 3 As in Fig. 1 20nm spherical particles and one 27nm particle penetrated by stain. Magnification 180000 ×

It was investigated whether the ultra-centrifugated pellet would produce precipitation lines with post hepatitis sera without Tween 80 treatment. Fig. 6 demonstrates that this was actually the case. The untreated pellet caused a line of total identity with the detergent treated specimen, although the line was weaker Fig 7 illustrates that both the treated and untreated pellets still contain HB determinants in amounts sufficient to give precipitation lines when tested by the common CHI method for HBAg detection.

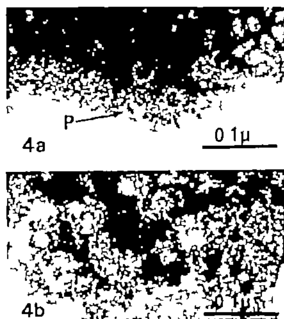


Fig 4 a + b Released 27nm particles aggregated by antibody Prepared from serum of a patient with chronic hepatitis (O.A.S.) treated with 5 per cent Tween 80 and reacted with post hepatitic serum. Magnification 180000 ×

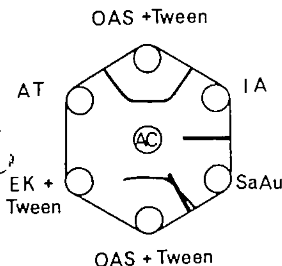


Fig 5 Closed hexagonal immunodiffusion showing non-identity of the 27nm antigen-antibody system to the common HB antigen-antibody system

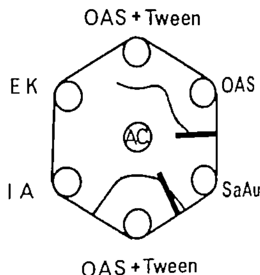


Fig 6 Closed hexagonal Immunodiffusion demonstrating 27nm antigen in O.A.S. serum without Tween 80 treatment.

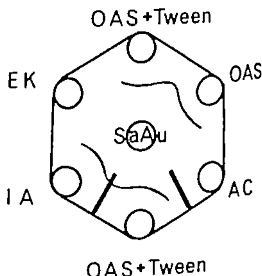


Fig 7 Closed hexagonal immunodiffusion illustrating persistence of HBAg after Tween 80 treatment and ultracentrifugation.

DISCUSSION

This paper reports the detection of a new antigen-antibody system intimately associated with virus B hepatitis. The antigenic determinant(s) of this system are totally different from HBAg determinants as demonstrated by the gel precipitation test (CIII)

There is a good reason to assume that the antigenic components of this new system are connected with the 27nm core released from the Dane particle by detergent treatment (1 14) In the first place an HBAg positive serum which does not contain Dane particles detectable by electron microscopy

does not give a positive gel precipitation in the new system. In the second place, a serum which does possess Dane particles and what appears to be free "cores" is producing precipitation lines irrespective of detergent treatment, although such treatment results in stronger reactions. In the third place, in the electron microscope, positive specimens do not demonstrate any definite structures other than the common HBAg particles and the 27nm "cores". It might be argued that a third component might be present as well. It does not seem likely however that particles occuring in numbers below the detection level of electron microscopy should produce visible precipitates in CHI. Since our sources of antigen at present are very limited, it has not been possible to go through any extended serological screening programme. It is interesting to note anyway that individuals who have been infected with virus B and are in quite different situations with regard to HBAg, all have antibodies directed towards the new specificities. This applies to a long term HBAg carrier serum, an HBAb positive serum and sera which contain neither HBAg nor HBAb. These findings are in striking contrast to the frequency with which HBAb are detected and to the amounts of these antibodies commonly present in sera (1). The latter statement is based on the fact that HBAb very seldom occur within the detection limits of the gel precipitation test CHI.

The question arises whether the antibodies detected in this new system represent a more constant immunological response to virus B than the HBAb. This study points to a possible explanation of the discrepancy in immunological response to the two hepatitis associated antigens. The fact that particles resembling the 27 nm "cores" of the Dane particles have been demonstrated in a serum does not prove definitely that they have been circulating as such *in vivo*. The core particles may possibly have been released from its coat after the blood specimen was drawn. But if it is accepted that 27nm particles at some time during a virus B infection is circulating freely it seems logical that the different immunological host response to the "cores" and the HB specificities may be due to a difference in host reactivity the cores being more antigenic compared with the common HBAg. Another explanation of course is that the exceptionally high particle number of an HB antigenemia causes some sort of an immunological paralysis. If so however it is very difficult to explain why multipletransfused individuals are such good producers of HBAb (12). After all it may be adopted that while HBAb most commonly are not present in post-hepatitis sera, or can only be detected by the aid of very sensitive techniques (6) the antibodies of the new system are present at levels expected for viral infections since they are traceable by gel precipitation technique.

At present there is no evidence that any morphological type of the particles demonstrated in connection with virus B hepatitis actually is the infectious agent of this disease. Nevertheless, some observations may indicate that the intact Dane particle and the 27nm "core" are "virion related". The morphology (4 1 14) the size (10) the number of particles in sera (11) the immunological response (1) and the positive staining with uranyl acetate (5) indicate strongly such a relationship applying specially to the 27nm "core" particle.

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CASEIN INDUCED AMYLOIDOSIS IN THE NUDE MOUSE

I *Acceleration of Amyloidosis in Recipients of Spleen Grafts from Casein-Sensitised Donor Mice*

II *Transfer of Amyloidosis by Spleen Cells*

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Amyloidosis was induced by repeated casein injections in a strain of mice with congenital aplasia of the thymus (nu/nu-C3H). The development of amyloidosis in this strain was delayed compared with the development of amyloidosis in the strain of control mice C3H. Spleen grafts from casein sensitized non-amyloidotic "nude" and C3H mice were transferred to mice from both strains. Only grafts from casein-sensitized C3H mice could accelerate amyloid formation in recipients, whereas grafts from the casein-sensitized "nude" mice had no such effect. Antibodies to casein could be detected neither in the donor mice nor in the recipients. The observed acceleration could be due to a transfer of casein stimulated T lymphocytes. Spleen cells from amyloidotic "nude" and C3H mice were transferred to both strains. Amyloid formation occurred only in recipients belonging to the C3H strain. As amyloid formation in the recipients—in this transfer model—is dependent on heavy cytotoxic treatment, it seems unlikely that amyloid formation in the recipients is due to immune reactions elicited by donor or recipient lymphoid cells. The reason why amyloidosis cannot be transferred by spleen cells to nude mice could be due to a poorer trapping of donor spleen cells in the nude spleens than in the normal C3H spleens.

Clinical and experimental data indicate a relationship between a suppression of the immune apparatus and the development of amyloidosis. Some authors have suggested that the immune defect is localized in the compartment of the immune apparatus concerned with cell-mediated immunity (*Ranløv & Jensen* 1966, *Cathcart et al.* 1970, *Hardt & Claësson* 1971, 1972 a). *Hardt & Claësson* (1972 a) suggested that extensive stimulation by antigen (casein) causes a decay of T lymphocytes primarily. Subsequently a factor is released from the dying lymphocytes which induces the macrophages to produce the amyloid substance.

This hypothesis, however, has been questioned, as *Hardt & Claësson* (1972 b) were able to induce amyloidosis in mice with a congenital aplasia of the thymus (nude mice) by prolonged casein stimulation. In the present study we have further studied the development and transfer of casein-induced amyloidosis in nude mice in order to elucidate the role of T lymphocytes in experimental amyloidosis.

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TABLE 1 *Serum Antibodies to Casein a d Spleen Amyloidosis in C3H and Nu/Nu-C3H Mice Durg Casein Stimul tion*

	No. of animals	No. of casein injections	Anti-casein antibodies	Spleen amyloidosis incidence	grade
C3H mice	6	20	0	6/6	3-4
	9	40	+ +	9/9	4 3
n /nu-C3H	7	20	0	0/7	0
	7	40	0	7/7	2 3
	6	60	(+)	6/6	2 3

MATERIAL AND METHODS

Two stocks of mice were used (obtained from the Laboratory Animal Breeding and Research Centre, Gl. Bomholtgård Laven, Denmark) 1) Mutation nudes (nu/nu-C3H) which had been bred using a cross intercross back cross system and had obtained 87¼ per cent synergy with the inbred strain of C3H mice used nu/nu (1-⅛)*C3H. 2) Highly inbred C3H mice served as controls.

After arrival in our animal room the mice were kept under clean conditions and fed sterilized mouse pellets. All mice were about 2 months old at the beginning of the experiments and sex distribution was equal.

Antigen. Sterilized casein was used in a 5 per cent solution in 0.25 per cent NaOH. The casein was sterilized in a 10M V linear electron accelerator at EAC, Research Establishment, Risø. The casein solution was given subcutaneously 5 times a week for different periods (conf. T ble 1)

C culating ntibodies to asein. Aliquots of 15 microlitre of serum from individual mice were tested for the presence of anti-casein antibodies using a modified Mancini technique (For further details see *Clausen & Hardt* (197 b)) The amount of antibody was quantitated by visual inspection of individual areas of precipitation and scores were estimated from the weakest [(x)] to the strongest [+ + +] reaction

T nsfer I nu/nu-C3H donor mice were given 10 injections of casein (conf. Table 2) and slices from the spleens were removed from donors anaesthetized with A ertin® and immediat ly transferred to the lateral margins of the right kidney of the anaesthetized recipient mice according to the method described by *Wheeler et al.* (1966) The remainder of the spleens were taken for histological examinations. The recipients—nu/nu C3H and C3H mice—received either a nu/nu-C3H spleen transplant or a C3H spleen transplant. The recipients received 10 injections of casein after the transplantation. All animals were killed with ether the day after the last injection of casein The right kidney carrying the graft, the left kidney the spleen and the liver were fixed in neutral formalin and embedded in paraffin. Sections were prepared as described below

Transfer II Donors were nu/nu-C3H and C3H mice that had received 40 injections of casein (conf. Table 3) The day after the last casein injection the animals were killed with ether and spleen cell suspensions were prepared by homogenizing the spleens in a Potter Elvehjem and washing the cells three times in ice-cold Hanks balanced salt solution. The final suspension was adjusted with Hanks solution to a concentration of 100 × 10^6 nucleated cell/ml. Small specimens were taken from the spleens and fixed in neutral formalin for histological examination. Recipients—nu/nu-C3H and C3H mice—received either 100 × 10^6 nu/nu-C3H spleen cells or 100 × 10^6 C3H spleen cells i.v. All recipients were subcutaneously injected three times w th 0.05 mg of nitrogen mustard (Erasol®) on day 1 3 and 5 after the cell transfer The day after the last injection of nitrogen mustard all recipients were killed. Spleen, liver and kidney were fixed in neutral formalin and embedded in paraffin Sections were prepared as described below.

As it has been questioned whether washed spleen cell suspensions from amyloidotic mice contain casein an additional experiment was carried out. Heavy amyloidotic mice (40 injections of casein) were injected subcutaneously with 0.2 ml of tritium labelled casei (0.17 mCi per mouse NEN Chemicals GmbH) 24 h before sacrifice. Spleen cell suspensions were prepared and washed as mentioned above and 100 × 10^6 cells concentrated in 100 µl buffered sal ne and counted in 5 ml Diovan scintillation fluid using a Beckman LS-100 C scintillation counter

Histology Sections were cut 5 micron thick and stained with haematoxylin-eosin, methyl green pyron ne, alkaline Congo red, and the PAS rea. Amyloid was identified by its morphology and its birefringence with Congo red under crossed polars. The degree of amyloidosis, if any, was evaluated in sections of spleens on a scale ranging from 0 to 6, ccording to the semiquantitative method described by *Ch stensen & Hjort* (1959

TABLE 2. *Amyloidosis in Donor Spleen Grafts and Spleens of Recipient Mice Treated with 10 Injections of Casein after Transfer of Donor Spleen Grafts*

Donors strain	Treatment of donors: No. of casein inject.	Recipient strain	No. of recipients	Amyloidosis in donor spleen graft: incidence	Amyloidosis in donor spleen graft: grade	Amyloidosis in recipient spleen: incidence	Amyloidosis in recipient spleen: grade
C3H	10	nu/nu-C3H	5	4/5	–	3/5	3–3
C3H	10	C3H	5	5/5	–	4/5	3–4
nu/nu-C3H	10	nu/nu-C3H	7	0/7	–	1/7	1
nu/nu-C3H	10	C3H	6	0/6	–	1/6	3

TABLE 3. *Amyloidosis in the Spleen of Recipient Mice Treated with 3 Injections of Nitrogen Mustard after Transfer of Donor Spleen Cells*

Donor strain	Treatment of donors: No. of casein inject.	Recipient strain	No. of recipients	No. of spleen cells transferred per recipient	Spleen amyloidosis: incidence	Spleen amyloidosis: grade
C3H	40	nu/nu-C3H	8	100×10^6	0/8	0
C3H	40	C3H	4	100×10^6	4/4	1–4
nu/nu-C3H	40	nu/nu-C3H	8	100×10^6	0/8	0
nu/nu-C3H	40	C3H	4	100×10^6	3/3*	2–4

* One mouse died during the nitrogen mustard injection period.

RESULTS

Table 1 shows the incidence and degrees of amyloidosis in the spleens of C3H and nu/nu-C3H mice after various doses of casein. As shown in previous studies (conf. *Ranløv* 1968) 20 injections of casein result in severe degrees of amyloidosis in normal C3H mice. In contrast, amyloid deposits were first observed after 40 injections of casein in the spleens of the "nude" mice. In both groups of mice, amyloid was found in a perifollicular position in the spleen. In the liver the amyloid was observed in the walls of the sinusoids and in the kidneys small amounts of amyloid were found in the glomeruli.

Antibodies to casein first appeared in the serum of normal mice after 40 injections of casein while detectable amounts of antibodies were found only in one "nude" mouse which had received 60 injections of casein.

Transfer I. All donor mice had received 10 injections of casein prior to the spleen graft transfer. At this time the spleens were characterized by perifollicular pyroninophilic proliferation which was most pronounced in the group of normal C3H donors. The results of 10 days casein treatment of the recipient mice are shown in Table 2. In all recipients, the spleen grafts showed a viable appearance without signs of inflammation. The transfer of spleen grafts from 10 days casein treated C3H donors to either normal or "nude" recipients followed by 10 days casein treatment of the recipients resulted in the development of amyloidosis both in the spleens and spleen grafts of the majority of the recipient mice. In contrast, only one normal and one "nude" recipient which had received spleen grafts from casein treated "nude" donors developed amyloidosis in their spleens and no amyloid was found in any of the spleen grafts from these groups.

Serum antibodies could not be detected either in the C3H spleen graft recipients or in the recipients of nu/nu-C3H spleen grafts.

Transfer II. The donors were amyloidotic

TABLE 4 *Counts per Minute in Three-time Washed Spleen Cell Suspensions Obtained from Normal and Amyloidotic C3H Mice Injected 24 Hours Prior to Sacrifice with 0.2 mg ³H-labelled Casein*

Counts per minute per 10⁸ washed spleen cells		
Background*	Normal C3H	Amyloidotic C3H
212 ± 32	410 ± 37	450 ± 68

* Spleen cells from C3H mice not injected with ³H-casein.

C3H or nu/nu-C3H mice which had received 40 injections of casein (Table 1) The results of the intravenous injection of 10⁸ donor spleen cells followed by treatment of the recipients with nitrogen mustard are shown in Table 3 All of the C3H recipients showed moderate degrees of spleen amyloidosis whether the donors were normal or "nude" mice. In contrast, none of the "nude" recipients showed development of spleen amyloidosis after transfer of spleen cells from amyloidotic C3H or "nude" donors.

Serum antibodies to casein could not be detected in any of the recipient mice.

From Table 4 it is seen that injected labelled casein was still present after 24 h in spleen cell suspensions used for transfer As the amount of injected ³H-casein is only 0 2 mg, the total amount of unlabelled casein in spleen cell suspensions from mice injected for 40 days with 0.5 ml of a 5 per cent casein solution may be considerably higher

DISCUSSION

In accordance with previous results from studies in nu/nu NMRI mice, (*Hardt* & *Claësson* 1972 b) 40 injections of casein induced amyloidosis in the nu/nu-C3H strain used in the present experiment In the above mentioned work randomly bred NMRI mice were used as controls. These mice however have a long amyloidosis induction phase (60 days) In the previous study we therefore suggested that the lack of T lymphocytes in the nude mouse leads to an acceleration of the amyloid formation. However in the light of the present results this suggestion must be revised as the strain of nude mice used here genetically is more comparable to the controls. Provided that lack of thymus is the sole aspect in which nu/nu-C3H differ from the parent strain, C3H, these results indicate that congenital lack of thymus—i.e. reduced capability to perform cell mediated immune reactions—results in a prolonged amyloid induction phase.

It has been shown that recirculating T lymphocytes are not present in the nude mouse (*Sprent* 1973) but other studies indicate that a small number of T-lymphocytes is present in the spleen and lymph nodes (*Raff* & *Wortis* 1970) The nude mice used in the present study had no θ-positive lymphocytes in the thoracic duct (unpublished data)

Thus, it is possible that small numbers of sessile T lymphocytes present in the lymphatic organs of the "nudes" could proliferate slowly due to casein stimulation. In this way the pyroninophilic phase in the spleen—which seems to be mandatory for the amyloid formation—is delayed, which again leads to a delay of the amyloid formation. Likewise, we have shown that a proliferation of T lymphocytes always precedes the amyloid formation and that the pyroninophilia in the spleens of the nude mice develops slower and never reaches an intensity in the nude mice as that found in normal mice (*Hardt* & *Claësson* 1972 a, b)

In contrast to our present results, *Ranlov* 1966 *Druet* & *Janigan* 1966, and *Ebbesen* 1971 have all showed that thymectomy prior to casein treatment results in a significant acceleration of the amyloid formation. The reason for this discrepancy cannot be explained.

In accordance with previous studies (*Hardt* & *Claësson* 1972 b, *Claësson* & *Hardt* 1972 a) we found pronounced amyloid formation both in normal and in nude mice whereas no antibodies against casein could be detected in the serum from these mice It was not until many casein injections had been given,

and the animals had severe amyloidosis, that increasing amounts of circulating antibodies to casein could be demonstrated. This clearly indicates that the humoral part of the immune system is functioning in these severely affected animals.

The formation of amyloid in the nude mice could be due to a casein induced decay of a quantitatively small but—in respect of the development of amyloidosis—important number of T lymphocytes. Such a decay which both precedes and follows parallel to the amyloid formation was first observed by *Claësson* & *Hardt* (1972 b) in normal casein treated C3H mice.

Spleen grafts from casein stimulated normal mice could accelerate the casein induced amyloid formation in normal as well as in nude mice whereas spleen grafts from similarly stimulated nude mice did not have this effect.

The amyloid accelerating effect of sensitized lymphoid tissue was first described by *Hultgren et al.* (1967) and later confirmed by *Hardt* & *Ranløv* (1968) and *Hardt* (1971) using the same graft technique as in the present study. Whereas *Janigan* (1969) considers the accelerating of amyloidosis due to a factor without immunological specificity *Hardt* & *Ranløv* considered the acceleration effect due to a specific transfer of immunity towards casein, as *Hardt* (unpublished) found that treatment of the recipient with antigens other than casein did not lead to acceleration. The present results open up the possibility that the acceleration is due to transfer of cell-mediated immunity towards casein, since transfer of "nude" spleen grafts (which lack sufficient numbers of casein stimulated T lymphocytes) were not able to accelerate amyloidosis in the recipient. On the other hand, the acceleration of amyloidosis would have to be due to the proliferation of a very small number of T lymphocytes as the C3H spleen grafts—which at average represent about 10 mg spleen tissue = 10×10^{6} cells—are able to accelerate amyloidosis to the same degree in "nude" and in normal recipients.

In the present spleen cell transfer model and in previous studies by *Werdelin* & *Ranløv* (1966) *Ranløv* (1968) and *Hardt* (1971) amyloidosis was transferred by spleen cells from amyloidotic mice to normal recipients, but amyloid formation did not take place unless the recipients were treated with cytotoxic agents in the days after the transfer. A specific role of donor spleen cells in the sense of immunological reactions by inoculated lymphoid cells has not been established. The transfer of amyloidosis by amyloidotic spleen cells may merely be a result of inoculation of amyloid producing reticular cells and amyloid containing macrophages which during the intensive treatment with nitrogen mustard are killed and trapped in the recipient spleen. The presence of casein in the transferred spleen cell suspensions found in the present work suggests another possibility namely that the positive transfer of amyloidosis by means of spleen cell suspensions is due to a transfer of T lymphocytes and casein. The T lymphocytes will lodge in the recipient spleen and here undergo decay as a result of the applied cytotoxic treatment and release a factor which together with the transferred casein stimulate the macrophages in the recipient spleen to produce amyloid fibrils. The absence of amyloidosis in the recipient spleens of "nude" mice which received either amyloidotic C3H or "nude" spleen cells is difficult to understand. Perhaps the poorly developed white pulp of the "nude" spleen together with the very well differentiated red pulp (*Hardt* & *Claësson* 1972b) results in trapping mechanisms of the spleen of "nude" mice other than those of normal spleens.

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STUDIES OF HUMAN KIDNEY ANTIGENS

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Investigations of antigens from three clinically well perfused human kidneys ha e been performed using xeno-antibodies of rabbit. The kidney preparations contained immunogenic amounts of normal human serum proteins. After absorption with autologous hepar tissue there were no signs in the absorbed anti-renal sera of remaining antibodies to serum protein if tested in doubl diffusion in agar gel. If tested against these hepar absorbed anti-renal sera, the saline extracted renal specific antigens were found to be thermolabile, trypsinstable and pepsinstable. A kidney cortex extract obtained after heating at pH 2.0 revealed a further antigen cross-reacting with the thermolabile kidney specific antigens. N indication of individual specificity could be found in double diffusion in gel analysis. The different anti-renal sera were equally cytotoxic to different human lymphocyte suspensions. The hepar absorbed anti-renal sera showed weak or non-lymphocytotoxic reactions to different human lymphocyte suspensions.

tudies of tissue antigens, their species- and rgan specificity as well as their cross-reac ivity have been the subject of several investi- ations (1 2, 3 6, 8 10 19 23 17) The idney specific antigens are of special interest ecause of their ability to induce experimen- al glomerulonephritis when heterologous anti-kidney serum is employed as described in the classical work by *Masugi* in 1933-34 (21) The pathogenesis of human glomerulonephritis include a possibility that exogenous antigens may cross-react with human kidney antigens (7) Informations about kidney specific antigens have become even more important since immunological cross-reactivity between kidney antigens and beta haemolytic streptococci, and recently also *E coli* has been reported (14) On the basis of these findings the cross-reactions have been claimed to be at least partly responsible for the pathogenesis of glomerulonephritis and the scarring of pyelonephritis. Many of the problems concerning chemistry structure and biological significance (11 13 16) of the kidney specific antigens are still unsolved.

The aim of the present investigation was to study and characterize human kidney specific antigens by means of rabbit antisera, evoked by heteroimmunization with the same kidney and further to study the kidney antibody activity in these rabbits before and after nephrectomy The significance of the HL-A system for the antigen-antibody reactions employing the lymphocytotoxicity test was also examined.

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MATERIAL AND METHODS

Kidney Material

Three human kidneys with warm ischaemia times of 14 90 and 21 minutes were used for the preparation of renal antigens. The kidneys were flushed out and cooled according to the standard clinical method used in the transplantation pro-

gramme (4) Until further preparation the kidneys were stored at −60–70 C.

The kidneys were selected to exclude as much as possible the presently known cross-reactivity within the HL-A system (28) The donor of kidney A had bloodgroup O and the tissue type HL-A 1 11 W5 + 5 8, kidney B bloodgroup O and HL-A 2, W10 27(AJ) and kidney C bloodgroup A and HL-A 28, 3 W15(?)

Kidney Antigen Preparation

After thawing the kidney the cortex and the medulla were separated macroscopically These two preparations were designated human renal cortex (HRC) and human renal medulla (HRM) respectively Each preparation was cut into small pieces and homogenized for 10 minutes with two parts phosphate buffer (pH = 7.2 M = 0.015) at 10 000 rev/min in an Omnimixer (Sorvall) and centrifuged for 20 minutes at 12,100 × g The supernatants (HRC-sup and HRM-sup respectively) were collected. The sediments were homogenized for one minute in phosphate buffer at 10,000 rev/min. After washing in phosphate buffer thre times at 12 100 × g the sediments were suspended in phosphate buffer and sonicated for 10 minutes at 1 2 ampère (Raytheon) Centrifugation for 30 minutes at 12,100 × g gave a supernatant, designated HRC-Ul sup and HRM Ul sup, respectively and a sediment which after suspension in phosphate buffer was called HRC-Ul sed and HRM Ul sed, respectively

From the cortex of kidney A another sediment fraction was also prepared. After removal of the HRC-sup, the sediment was washed three times, suspended in phosphate buffer (total volume 12 ml) and homogenized for one minute at 10,000 /min. This preparation was designated HRC-A d From this sediment two preparations were e

1) A heat and acid stable antigen preparation was extracted using the extraction method for preparing polysaccharides from streptococci as described by *Lancefield* (18)

2) The extraction procedure for BE-kidney antigen of *Intorp & Milgrom* (15) was performed by extraction at +100 C followed by precipitation at 71 per cent ethanol concentration.

A glomerulus preparation processed acording to the method of *Greenspoon & Krakower* (11) was also made.

Heat and Enzyme Treatment

The antigenicity was also studied after heating and after treatment with trypsin and pepsin. All preparations, with the exception of the glomerulus preparation and those extracted according to the procedures of *Lancefield* (18) and *Intorp & Milgrom* (15) were heated for 30 minutes at +50 C, +80 C and +95 C. The trypsin exposure (1 mg/ml renal preparation) was performed at pH 8.0 for 60 minutes at +37° C. The reaction was stopped by the addition of soy-bean trypsin inhibitor in slight excess. Treatment with pepsin was performed at +37 C in 0.07 M acetate and 0.05 M chloride as sodium salts, and cysteine (pH = 4) for 1 and 5 hours. The pepsin concentration was 3 per cent of the protein content of the preparation. The digestion was interrupted by raising pH to 8.0

Preparation of Hepar Antigens

Liver tissue from the three donors was [illegible] with isotonic saline and stored at −20° C. After thawing it was cut into small pieces, suspended in phosphate buffer (pH = 7.2) at a tissue-concentration of 500 mg/ml, homogenized for 15 minutes at 10,000 rev/min and centrifuged for 30 minutes at 12,100 × g Floating fat, if any was removed. The supernatant was designated HL-sup. The sediment was homogenized for one minute at 10,000 rev/min. After washing twice in phosphate buffer the sediment was suspended in phosphate buffer and sonicated in the same way as the renal sediment. The sonicated preparation was centrifuged and separated in supernatant called HL Ul sup and after suspension in 20 ml phosphate buffer the sediment was called HL Ul sed. All the preparations of renal and hepatic origin were inoculated on blood-agar plates to verify bacterial sterility. All preparations were stored at −20° C.

Immunization

The preparations of renal tissue were injected into adult New Zealand white rabbits. Each preparation was administered 4–5 times every 10th day to two rabbits. The sonicated sediment preparations were given intravenously to the same rabbits that received the sonicated supernatant subcutaneously with Freund's complet adjuvant (Difco) The other preparations were injected subcutaneously with Freund's complete adjuvant. HRC-A-sed was injected subcutaneously with complete adjuvant but also diluted in phosphate buffer (dilution 1 20) and injected intravenously into the same rabbit. When the immunization procedure was finished the rabbits were nephrectomized bilaterally Serum samples were taken before immunization, before nephrectomy and 24 hours after nephrectomy During the operation, urine was collected by puncturing the bladder Inoculation on blood-agar plates did not reveal any bacteriuria

Absorption

The renal antisera were absorbed with an equal amount of pooled normal human serum for 1 hour by gentle shaking at +37 C. The materials were

TABLE 1 *Number of Precipitates with the Different Renal Antigens and the Corresponding Renal Antibodies (Rabbit)*

Renal antigen	Rabbit antiserum of renal antigen			
	anti HRC sup	anti HRM sup	anti HRC UI (sup + sed)	anti HRM UI (sup + sed)
HRC sup	6	5	5	4
HRM sup	6	5	5	4
HRC (sed)				
UI sup	3	1	2	3
UI sed	5	3	3	3
HRM (sed)				
UI sup	2	1	2	2
UI sed	5	3	2	2 (3)

centrifuged for 20 minutes at 4,500 × g and the supernatants were tested.

Every renal antiserum was absorbed with its corresponding three different preparations of autologous hepar. Each absorption meant gentle shaking for 1 hour at +37 C and centrifuged for 20 minutes at 4,500 × g. The final supernatant was designated hepar absorbed anti-kidney-serum.

Techniques

The protein contents of the different preparations were determined according to the method of *Lowry et al.* (20). The differences in protein content in the corresponding antigenic preparations of the kidneys and the livers were small.

Immunodiffusion analysis of the renal antisera and the different antigenic preparations were investigated in agar gel in a microplate technique (29) which is a modification of the method of *Ouchterlony* (25) as well as by means of immunoelectrophoresis (30). The lymphocytotoxicity of the different sera was examined against number of different human lymphocyte suspensions (17). As negative controls served sera from three non-immunized rabbits and normal human AB-serum. Rabbit complement was used and this was stored at 70° C.

RESULTS

Kidney

If the six different antigenic preparations from kidney A, B and C were compared in immunodiffusion with anti-HRC-A sup anti-HRC-B sup and anti HRC-C sup respectively the sonicated sediment fractions (HRC-UI sed and HRM UI sed) showed all but one precipitation lines in common with HRC-sup and HRM-sup. The two latter preparations showed one more precipitinogen that was common to these two fractions.

Any precipitinogens specific for the sonicated preparations could not be found and it applies to all three kidneys that the UI sup preparations showed weaker and fewer precipitinogens than the UI sed preparations.

A similar pattern was found when the different antigenic preparations were examined against anti-HRM-sup.

The different sonicated antigens did not show any specific precipitation lines to anti HRC-UI (sup + sed) or anti HRM UI (sup + sed) when these antigens were compared with the reactions of the non-sonicated preparations. The findings in kidney A are illustrated in Table 1

Serum samples obtained from the rabbits before immunization, after immunization but before nephrectomy and on the day after nephrectomy were tested with the different antigenic preparations from all three kidneys. There were no precipitins in the pre-immunization sera. No precipitins were revealed specifically in the sera collected after nephrectomy as compared with the pre nephrectomy sera.

Cross-reactivity with Hepar Antigens

Several precipitinogens were found to be common to the renal antigenic preparations and the hepatic preparati[illegible] the [illegible] donor if tested with the [illegible] anti-kidney [illegible] sera. But precipitino[illegible] specific [illegible]

gramme (4) Until further preparation the kidneys were stored at –60–70 C.

The kidneys were selected to exclude as much as possible the presently known cross-reactivity within the HL-A system (28) The donor of kidney A had bloodgroup O and the tissue type HL A 1 11 W5 + 5 8, kidney B bloodgroup O and HL-A 2, W10 27(AJ) and kidney C bloodgroup A and HL-A 28, 5 W15(?)

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2) The extraction procedure for BE-kidney antigen of *Interp & Milgrom* (15) was performed by extraction at +100° C followed by precipitation at 71 per cent ethanol concentration.

A glomerulus preparation processed acording to the method of *Greenspoon & Krakauer* (11) was also made.

Heat and Enzyme T eatme t

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Absorption

The renal antisera were absorbed with an equal amount of pooled normal human serum for 1 hour by gentle shaking at +37° C. The materials were

TABLE 2. *Number of Precipitates with the Different Renal Antigens (Human Kidney C see Text) and the Different Renal Antibodies (Rabbit) Absorbed with Aut logous H par Antigens*

Renal antigen	Rabbit antiserum of renal antigen after absorption with autologous hepar antigens			
	anti HRC sup	anti HRM sup	anti HRC Ul (sup + sed)	anti HRM Ul (sup + sed)
HRC sup	4	2	2	
HRM sup	4	2	2	2
HRC (sed)				
Ul sup	2	0 ?	0	0
Ul sed	4	2	2	1
HRM (sed)				
Ul sup	0	0	0	0
Ul sed	1	0	1	0

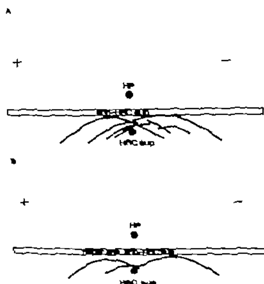
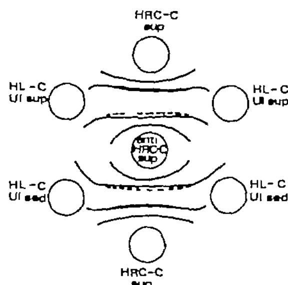


Fig 3 Immunoelectrophoretic comparison of a kidney antigen (HRC sup) and pooled human serum (HP) with the corresponding anti-kidney serum (A) and the corresponding hepar absorbed anti-kidney serum (B)

sera of the donors A and C, but analyses of hepar absorbed anti HRC-B sup and HL-B sup revealed a weak precipitation line. Similar indications of precipitins could also be found if the absorbed anti HRM B sup, anti-HRC-B Ul (sup+sed) and anti-HRM B Ul (sup+sed) were used. This indicated an incomplete absorption of the anti kidney sera with autologous hepar from these donor B. With one exception, no precipitates were found in the analyses of the hepar absorbed anti kidney sera with human normal sera. Hepar absorbed anti HRC-A sed produced one weak precipitation line and the unabsorbed anti-HRC-A sed showed 9 precipitins with human normal sera. The different hepar absorbed anti-kidney sera wer examined with the corresponding renal antigenic preparations. The strongest reactions were observed in cases of the non-sonicated renal preparations and their corresponding hepar absorbed anti-sera.

The HRM-sup preparations and hepar absorbed anti-HRM-sup situation caused one precipitate for kidney A, B and C respectively. The sonicated renal Ul-sup preparations showed generally weaker and fewer precipitates with the corresponding absorbed antisera than the Ul-sed preparations and their corresponding anti-sera. The number of precipitinogens was not greater in kidney B in different situations than in kidney A or C. As regards kidney C, the number of precipitates in different situations is illustrated in Table 2.

The different hepar absorbed renal antisera of HRC sup A, B and C were tested against the glomerulus preparations and all showed one precipitation line. This precipitation line was not cross-reacting with the antigenic HRC sup preparations heated to +80 C or those heated to +95 C. The glomerulus preparation revealed two precipitinogens with anti HRC-A sup two with anti-HRC-B sup and one precipitinogen with anti-HRC-C sup If this precipitation spec



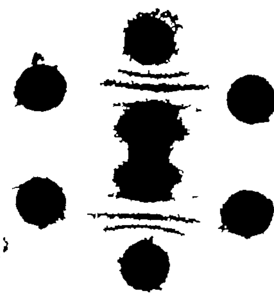


Fig 1 Comparison between one renal preparation (HRC sup) and two different sonicated preparations of hepar (HL UI sup, HL-UI sed) (from the same individual) tested against the corresponding anti kidney serum. Two kidney-specific precipitates are demonstrated.

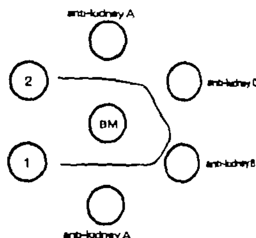




Fig 2 Autologous hepar absorbed anti-HRC-UI (sup + sed) from kidney A, B and C [illegible] to the glomerular preparation (BM) and compared to pre immunization sera of HRC-A sup (1) and HRC-A UI (sup + sed) (2)

for the kidney preparations were also found (Fig 1)

Absorptions

Every renal anti-serum was tested in double diffusion in gel against human normal serum. One or two, and in one situation three faint precipitates could be seen with the different renal anti-sera. Absorption of the renal anti-sera with human normal serum and centrifugations did not change the number of precipitates, as compared with the unabsorbed anti-sera tested against the corresponding renal antigens. After absorptions of the renal anti-sera with the autologous liver preparations these were checked for remaining cross-reactivity against the autologous liver preparations. No precipitins could be detected in the hepar absorbed anti-kidney

TABLE 4 The Number of Precipitates Between the Different HRC Sup P eparations from Human Kidney A B and C Heated at Different Temperatures for 30 Minutes and) their Corr sponding Anti-Renal (Rabbit) Sera b) the Cor esponding Anti-Renal Sera Absorbed with Autologous Hepar Tissue

	not heated	+50 C	+80 C	+95 C		
HRC-A sup	6	6	4	1	anti-HRC-A sup	)
HRC-B sup	6	6	4	3	anti-HRC-B sup	
HRC-C sup	6	5	4	1	anti HRC-C sup	
HRC-A sup	2	2	0	0	absorbed anti-HRC-A sup	b)
HRC-B sup	5	2	0	0	absorbed anti-HRC-B sup	
HRC-C sup	4	2	0	0	absorbed anti-HRC-C sup	

antisera of kidney C. None of these precipita tion arcs could be referred to transplantation antigens as absorption of a kidney antiserum with a homologous kidney antigen preparation left no precipitin in the antiserum if this was tested against nologous hepar and kidney antigen preparations. The number of precipitation arcs was reduced after absorption with nologous hepar and only three precipitation bands were seen after immunoelectrophoresis (Fig. 3 B)

Absence of Individual Specificity

The corresponding kidney fractions from the different kidneys were compared with one another in double diffusion against all the corresponding absorbed anti-sera. No indica tion of precipitinogens specific for any of the kidneys could be found. The number of pre cipitinogens were nearly the same in the corresponding precipitation situation. The small differences are illustrated in Table 3 A and 3 B. The differences were probably ex pressions of differences in optimal concentra tions for precipitation and could not be ascribed to individual specificity

Heat Sensitivity

The different HRC sup preparations from the different kidneys were treated for 30 minutes at +50 +80 and +95 C. They were tested against the corresponding anti-renal sera. The number of precipitates were reduced (Table 4) as compared to the untreated renal antigenic preparations. The preparations were also tested against the cor responding renal anti-sera, absorbed with autologous hepar representing the renal spe

TABLE 5 Th Resulting Reactions in Gel-D ffusion After Antibody Blocking by P diffusion Between the H at Eluated Antigenic F action of HRC A Sup and HRC-A S d and Pooled Human Serum Respectively

Antigen	Antibody blocking* Antigen preparation	Antiserum	Resulting precipitate
HRC-A sup heated (+95°C)	HRC-A sup heated (+95°C)	pooled human serum	1
		absorbed§ pooled human serum	1
HRC-A sed heated (+95 C)	HRC-A sup heated (+95 C)	pooled human serum	1
		absorbed§ pooled human serum	1
	HRC-A sed heated (+95°C)	pooled human serum	0
		absorbed§ pooled human serum	0

* Performed by prediffusion for 3 hours with the different antigenic preparations.
§ Absorbed with thoroughly washed human AB-erythrocytes for 30 minutes at +37 C.

cific antibodies. No precipitates could be seen using the antigenic preparations that were heated to +80 C and +95° C, while the unheated and the preparations heated to +50 C showed two to four precipitation lines. *No precipitates could be found if the* antigenic preparation heated to +80 C was tested against normal human serum.

Two parts of HRC-C-Ul sed and one part of HRC-C-Ul sup were mixed and heated in the same way as described above. The number of precipitates were reduced from 4 using the unheated fraction, to 3 3 and to 1 if the different fractions heated to +50 +80 and +95 C, respectively were compared with the renal anti-serum of HRC-C-Ul (sup +sed)

When HRC-A sed was heated in the same way as the HRC-A sup a reduction in the number of precipitation lines was found when tested against the anti-HRC-A sed. No precipitation line could be found when the antigenic sediment fraction was heated to +95 C and investigated with the hepar absorbed anti HRC-A sup and anti HRC-A sed, respectively However this heated antigenic preparation showed a precipitinogen with *pooled normal human serum. This precipitin* in the pooled serum was still present after absorption for 30 minutes at +37 C with an equal amount of concentrated, washed AB(+)-erytrocytes. Further investigation of the HRC-A sed heated to +95 C was performed by means of antibody blocking with prediffusion for three hours with HRC-A sup and HRC-A sed, respectively both heated to +95 C. *It was not possible to prevent the* antibody-antigen reaction in gel between HRC-A sed (+95 C) and pooled human serum by prediffusion with HRC-A sup (+95 C) If however the prediffusion was performed with HRC-A sed (+95 C) no precipitation line would be visible (Table 5)

In order to further characterize the kidney antigens, a thermostable BE-preparation produced *as described by Ixtorp & Milgrom* (15) was analysed in the immunodiffusion system with the different renal anti-sera and compared with the different kidney antigen preparations. No precipitation line between the BE-preparation and the kidney anti-sera was seen. Furthermore the kidney-anti-kidney precipitation lines did not show any deviation towards the well containing BE-preparation.

*In repeated experiments with the Lance-*field preparation (18) of the kidney sediment from the cortex at least one precipitation line formed between this antigenic preparation and hepar absorbed anti-HRC sera. This reaction was seen most clearly in the case of hepar absorbed anti-HRC-C sup. This line fused with one of the lines in the HRC sup–anti HRC sup precipitation spectrum by a reaction of partial identity Absorption of the sera with AB(+)-erytrocytes did not abolish this cross-reaction

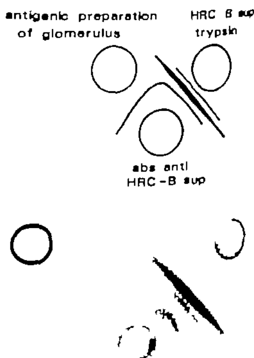


Fig 4 The trypsin digested HRC-B sup and the glomerul preparation compared and tested to anti-HRC-B sup absorbed with autologous hepar

ABLE 6. *The Lymphocytotoxic Reaction of the Different Anti-Kidney Sera (Rabbit) Against Different Lymphocyte Donors (Human)*

idney	Anti HRC sup			Anti HRM sup			Anti HRC Ul (sup+sed)			Anti HRM Ul (sup+sed)		
	A	B	C	A	B	C	A	B	C	A	B	C
L A-lymphocyte												
… 8, BB	4	4	4	4	2	2	4	4	2	4	2	2
Ba LND 8L	3	3	4	4	0	1	4	4	1	3	2	1
10. LND AJ	4	4	4	4		2	4	4	2	3*	1	1
11 12, FJH	4	2	4	4		1	4	3	0	3	2	1
LL 7 LND	4	3	4	4	0	2	4*	4	1	3*	1	2
BB, LND	4	4	4	4	2	3	4	4	1	4*	2	?
LL 7 12	4	4	4*	4	2*	3*	4	4	2*	4	2*	3*

he figures with asterisk indicate situations where the lymphocytotoxic reaction should have been nega-ve according to the HL-A system. Positive cytotoxic reaction was graded 1 to 4. The number of killed mphocytes was < 10 per cent in the negative reaction, 10–20 per cent in grade 1, 20–50 in grade 2, 0–75 in grade 3 and > 75 per cent in grade 4 of the positive reaction.

Enzyme Susceptibility

Trypsin. Trypsin digestion of HRC sup preparations of kidney A, B and C resulted 2, 3 and 2 precipitinogens, respectively if vestigated with their corresponding renal ti-HRC sup sera absorbed with autologous epar tissue. Identity reactions were seen if e normal HRC sup preparations from the dneys A, B and C were used, indicating that e renal specific antigens were stable to ypsin digestion. One or possibly two of the ecipitation lines with trypsin digested IRC-B sup and the corresponding hepar bsorbed serum showed no reaction of identity with the glomerulus preparations (Fig. 4). The precipitinogens of the other two kidneys, f used in the same way also indicated non-dentity reactions. All three absorbed anti-HRC sup sera showed one precipitate with he glomeruluspreparation. The precipitation attern changed if the original HRC sup reparations were tested against the anti-HRC sup and compared with the trypsin digested HRC sup preparations, but the differences were not greater than those to be explained by differences in optimal concentrations, which might indicate an uncomplete trypsin digestion.

Pepsin. The susceptibility to pepsin of the HRC sup preparations from kidney A, B and C, respectively resulted in a reduction in the number of precipitation lines. The pepsin treated (5 hours) and the trypsin treated HRC-A sup preparations showed two identical precipitates if tested against the corresponding hepar absorbed renal anti-serum. In the same situation, kidney B showed two identical lines and another faint line reacting only with the trypsin digested fraction. As regards kidney C, two identical lines were also seen, but the trypsin digested preparation presented in all four precipitation lines.

If the different pepsin digested preparations were compared with the glomerulus-preparation and investigated with hepar absorbed anti-HRC sup of the three kidneys, respectively all lines showed identity and it was possible to find precipitates between the glomeruluspreparations and the hepar absorbed anti HRC sup of kidney B and C. Consequently it seemed as if the renal specific antigens remained unaffected of pepsin digestion.

Lymphocytotoxicity

The different anti-kidney sera were tested for cytotoxicity using seven non-related, human lymphocyte suspensions. The reactions

were graded according to the percentage of killed lymphocytes. Less than 10 per cent killed cells were regarded as a negative reaction and the strongest positive reaction was associated with more than 75 per cent killed lymphocytes. No positive reactions could be found if the control sera were used. Table 6 illustrates the differences in lymphocytotoxicity of the renal anti-sera. Each serum seemed to be cytotoxic to the same degree, irrespective of the origin of the lymphocyte suspension. The figures with asterisk (Table 6) indicate situations where the cytotoxicity reactions should have been negative according to the HL-A system. Anti HRC-A sup and anti-HRC-C *sup showed more than 50 per cent* killed lymphocytes in titres 1/9 and gave a negative lymphocytotoxic reaction in titre 1/81 and 1/27 respectively if investigated with lymphocytes from two non-related individuals. Renal antisera absorbed with autologous hepar were tested for lymphocytotoxicity as above. The sera after absorption showed negative reactions or at most a killing of 10 per cent of the different lymphocyte suspensions, with the exception of anti HRC-B-UI (sup+sed). This serum constantly showed a positive reaction including a killing of 20–50 per cent of the lymphocytes irrespective of the HL-A system. No similar reaction could be found if the antisera of kidney A or C were used. If a six times thoroughly washed hepar sediment replaced the three hepar antigenic preparations to absorb anti HRC-A sup a killing of about *10* per cent of *the* lymphocytes was achieved with lymphocytes which according to the HL-A system were not cross-reactive with the antigens of the kidney. Using HL-A cross-reacting lymphocytes, a killing of about 20 per cent was seen, but the serum was noncytotoxic in titre 1/3 of this absorbed anti-HRC-A sup.

The kidney specific antibodies were found in double diffusion in gel to be non-specific for the individual donor. This observation was not contradicted by the lymphocytotoxicity studies. The absorption with autologous hepar preparations should have eliminated HL-A antibodies.

DISCUSSION

The present antigenic preparations of kidney cortex and medulla did not show any precipitinogens in immunodiffusion specific for cortex or medulla. Our studies showed five or six different precipitation lines with the non-sonicated antigenic preparations if investigated with the corresponding anti-sera. *Linder* (19) used the same method of hetero-immunization in rabbits with human kidney material. He showed eight precipitation lines in immunodiffusion. *Linder* however used pooled kidney homogenate for immunization. The warm ischaemia time of the kidneys exceeded *that of kidneys in our series*. Further, he made extensive serum absorptions with a view to eliminating reactivity to more widely cross-reacting antigens. The liver was among the organs that showed the highest number of cross-reactions (5, 19) with human serum absorbed anti-kidney sera. *Acerkina et al.* (1) also found eight precipitinogens in human kidney tissue investigated with anti-kidney sera, but 2–3 of these showed kinship to all organs and human serum and were thus antibodies to serum proteins. *Nairn et al.* (23) found three precipitinogens in gel diffusion between microsomal material of human kidney and anti-kidney serum of rabbit. Using a refined technique *Edgington et al.* (8) isolated a specific renal tubular antigen. In the present investigation, serum proteins in the kidneys or similar antigens were present in *quantities sufficient to* induce antibody production in spite of the intense perfusion of the kidney within 21 minutes post mortem.

The renal anti-sera absorbed with autologous hepar tissue presented a maximal number of 4 precipitins to the corresponding renal preparations. These antibodies were not cross-reactive with normal human sera and were designated kidney specific antibodies. The number of kidney specific reactions is similar to the number observed in different species by other investigators (5, 6, 19, 21, 27). Several investigations of human kidney antigens have been made. *Acerkina et al.* (1) found in gel-diffusion studies 2–3 kidney spe-

cific antigens. *Linder* (19) found at least four antigens to be present in the proximal secreting tubules after absorptions with different tissues. The antigenic preparation of renal tubular epithelium in rats (*Edgington et al.* 8) capable of producing experimental allergic glomerulonephritis, was cross-reacting with antigens from the brushborder of intestinal mucosa. Absorption of immune sera has disadvantages in diluting the immune sera. By absorption with human normal serum, some antigens which may be present in small amounts in normal human serum might remove antibodies to these antigens.

As cross-reactive iso-antibodies are known to occur within the HL-A system (28) these may have been present in the antisera of the renal preparations of kidney A and C, and of kidney B and C. Theoretically the antisera of kidney A and B may have presented some differences as the transplantation antigens are not cross-reactive according to the present knowledge of cross-reacting HL-A antibodies. Any indication of such differences could not be found in this investigation. The immune sera corresponding to the sonicated antigenic preparations probably contain HL-A antibodies, but evidently not in amounts sufficient to be detectable by the double diffusion in gel method. The possible HL-A antibodies ought to have been removed if absorbed with the autologous hepar tissue. This was also supported by the reactions in the lymphocytotoxicity tests.

The heat sensitivity of the renal preparations were investigated by means of the hepar absorbed anti-renal sera. No thermostable kidney specific antigens were found which is in contrast to findings by *Intorp & Milgrom* (15) who reported that their kidney specific antigen was stable to heat at +100 C. The renal antigenic material in the present report did not show any precipitates with hepar absorbed anti-renal sera after heating to +80 C. After heating of the sediment preparation from renal cortex to +95 C this investigation indicated the presence of a new precipitinogen in tests against pooled human normal serum. This precipitinogen did not react with the renal antiserum that had been absorbed with autologous hepar tissue. However the BE-preparations have been reported to be species specific (22). Using the Lancefield extraction procedure, an antigen was found in the kidney cortex sediment preparation which must be considered thermostable. It is worth noting that this heat stable antigen cross-reacted in immunodiffusion analysis with one of the heat labile kidney specific antigens. The immunodiffusion analysis (partial identity) indicated the possibility of common antigenic determinants, one of these being heatlabile, the other heatstable.

The glomerulus preparation tested initially gave an impression of two precipitation lines visible in different situations with anti-renal sera. Comparison with the hepar absorbed anti-renal sera however presented superimposed lines.

Because of the reported high incidence of cytotoxicity of normal rabbit serum for human lymphocytes (9, 12) the pre immunization sera from the rabbits used for induction of antibodies were tested, a further three normal rabbit sera were also tested. Using the here described cytotoxicity method we were unable to demonstrate any natural xeno-antibodies in 15 sera. This is in contrast to *Herberman's* (12) finding of an incidence of 90 per cent and that of *Ferrone et al.* (9) 30 per cent of cytotoxic antibodies to human lymphocytes in sera from normal rabbits. The small dilution in our method may explain the absence of natural cytotoxic xeno-antibodies. *Rogentine* (26) used a dilution of 1:2 in his cytotoxicity investigations and this gave a low concentration of cytotoxic xeno-antibodies. Heat lability has been reported to be a common feature of these antibodies. *Herberman* also reported that repeated freezing and thawing resulted in complete loss of such xeno-antibodies. To exclude that our cytotoxic reactions could be caused by xeno-antibodies, three strongly cytotoxic antikidney sera were freezed and thawed several times and heated for 30 minutes at +56° C. No loss of cytotoxic activity resulted. No change in the cytotoxic titres could be found in tests

of heated and non-heated anti-HRC-A sup In all situations where the absorptions with the autologous hepar were complete, the remaining kidney specific antibodies had no cytotoxic effect on human lymphocytes. These results may indicate that the kidney antigens immunogenic to rabbits are missing as surface antigens on human lymphocytes. This needs further elucidation using methods more sensitive than the lymphocytotoxic reaction. The possible interaction of the hepar antigenic material or of remaining antigen-antibody complexes in the renal anti-sera absorbed with autologous hepar cannot be excluded in the lymphocytotoxic reaction. The results of the absorption with a thoroughly washed hepar sediment indicated that such interaction could be present but is of little importance. Further studies by which to clarify this, necessarily implies the induction of immunological tolerance.

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STAPHYLOCOCCAL RADIOIMMUNOASSAY FOR HEPATITIS B ANTIGEN AND ANTIBODY

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A rapid radioimmunoassay (RIA) for the measurement of hepatitis B antigen and antibody is described in which protein A carrying *Staphylococcus aureus* is used as a solid phase anti-gamma globulin reagent. The tests are easy to perform, they are completed in two hours, and are easily automatized. The competition principle is used for antigen testing while antibody is measured by direct binding of radiolabelled hepatitis B antigen. Using human reconvalescent antisera, the staphylococcal test for antigen in 25 µl serum has about the same sensitivity as the Ausria test. The staphylococcal test for antibody has at least the sensitivity of the passive hemagglutination test. Both tests are highly specific.

The radioimmunoassays presently in use for the demonstration of hepatitis B antigen (HBAg) and antibody (HBAb) are time consuming We describe here a rapid radioimmunocompetition test for HBAg and a radiolabelled antigen binding test for HBAb which may be completed in 2 hours. *Staphylococcus aureus* containing protein A is used in the test for the separation of bound antigen from not bound antigen (7 8, 3 10) The staphylococcal test has about the same sensitivity as the Ausria test for HBAg (11) It is highly specific and does not require neutralization tests for specificity (13) The staphylococcal test for HBAb has at least the same sensitivity as the passive hemagglutination test (17) and seems also to be highly specific. The tests have been used in cases of blood donors and patients for more than one year

MATERIALS AND METHODS

HBAg A human serum (JEB 10908, subtype ad) with a HBAg staphylococcal RIA titre of 40000 and with no detectable HBAb was used as the source of antigen. The HBAg was isolated and purified for labelling by the following procedure In the first step, the antigen was isopycnically banded, using a linear cesium chloride gradient ranging from a density of 1 1 to 1.6 g/cm³ (5) This was achieved by layering 6.5 ml clarified serum onto 6 ml CsCl-gradient and centrifuging 6 tubes at a time in the Spinco L2-65B ultracentrifuge using the SW-40 rotor at 4°C and 30000 rpm for 17.5 hours. The tubes were fractionated and the fractions containing HBAg-activity were pooled In step two the pool was gel filtrated on Sephadex G-200 using Sephadex column K-25/45 and PBS as elution buffer The first protein peak to be eluted, corresponding to the void volume, contained the HBAg-activity and was collected. In order to concentrate the antigen before the third and final step, it was spun down in a Spinco rotor 65 by 62000 rpm for 3 hours. The supernatant was discarded and the pellet was resuspended overnight in a small volume of PBS. In the third and final step, the antigen was rebanded isopycnically in a linear CsCl-gradient with density from 1 1 to 1.4 g/cm This time 0.5 ml of the resuspended pellet was layered onto the 12 ml gradient and centrifuged for 17.5 hours at 30000 rpm and 4 C using a Spinco SW-40 rotor The HBAg containing band was collected and dialyzed against PBS overnight. The protein content in the final HBAg-preparation

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was 0 96 mg/ml, the density before dialysis being 1.20 g/cm³

Labelling of HBAg with ¹²⁵I (6) The HBAg was conjugated with ¹²⁵I in a total volume of 375 µl. This consisted of 1 mCi Na¹²⁵I with a specific activity of 300–500 mCi/ml (NEN Code NEZ-033H) 50 µl 0.5 M phosphate buffer pH 7 4 200 µl HBAg (ca 180 µg) 25 µl chloramine T (100 µg) 50 µl $Na_2S_2O_5$ (240 µg) The oxydation process was run for 45 seconds before the addition of sodium metabisulphite. After the addition of 50 µl KI (1000 µg) the mixture was pplied to a Sephadex G-200 superfine column (Sephadex K16/40) The buffer used for elution was PBS with 0.5 per cent human albumin (Sigma Fraction V)

Antisera. The antisera used were RJH 19044 which came from a patient in whom liver cirrhosis was discovered and who inadvertently had been transfused with a HBAg containing blood unit (14) HMB was obtained from an accidentally discovered blood donor ES, from a patient with chronic active hepatitis and BH, from a healthy person. None of the persons HMB, ES, and BH had previously received blood transfusions. These sera can therefore be considered as convalescent antisera, and RJH 19044 possibly as a hyper immune serum.

Radioimmunoassay 25 µl of a serum to be tested were distributed in two tubes, one for HBAg and one for HBAb screening. For antigen detection, 25 µl of antiserum in a suitable dilution (RJH 19044 diluted 1/5000) were added t one of the tubes. Both tubes were incubated at 37 C for 30 minutes. Subsequently 10 µl of radiolabelled HBAg (ca 6000 cpm) was added to each tube and the tubes were incubated for 60 minutes at 37 C. After incubation, 1 ml of a suspension of *St phylococcus aureus* was added to each tube using a Cornwall pipette and the tubes were immediately centrifuged at 2000 rpm for 5 minutes in a Sorvall GLC-1 centrifuge. The supernatants were sucked off and the tubes with the sediment of bacteria were measured for one minute in a gamma spectrometer (Nuclear Enterprises 8311)

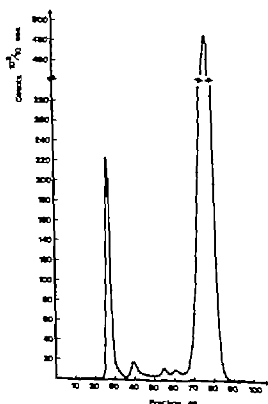
St phylococcus au eus. The strain Cowan I was cultivated in a modified "CCY" medium (2) at 37 C in Erlenmeyer flasks with indentations. After 15–20 hours of growth, the bacteria were harvested by centrifugation in MSE Mistral 6L centrifuge at 2000 rpm for 70 minutes The bacteria were then resuspended in a 2 per cent solution of formaldehyde in PBS, using half the original volume of the medium. The suspension was shaken in the incubator for another hour and half After this treatment, the bacteria were washed three times with PBS. Finally they were made up in PBS to a concentration of about 10 per cent (v/v) for standardization. This was done by using different dilutions of the 10 per cent suspension in the staphylococcal RIA test. The amount of bacteria sufficient to sedimentate the maximum number of counts with standard antiserum, as compared with a control consisting of a pool of 10 normal human sera, was used for screening of the sera. This was normally a 3 per cent (v/v) suspension.

Protein concentrations were estimated according to the method of *Lowry et al.* (12)

The staphylococcal strain was kindly supplied by P Oeding, Haukeland sykehus, Bergen, Norway

RESULTS

The elution diagramme after iodination is presented in Fig. 1 The first peak represents the void volume and contained the radiolabelled HBAg used in the test. A further three small peaks did not react. The low molecular iodine was contained in the last



F g 1 Sephadex G-200 Superfine chromatography of the reaction mixture after the radiolabelling process of the HBAg preparation. The column was eluated with PBS containing 0 5 per cent human serum albumine, and the eluate was collected in 1 ml fractions.

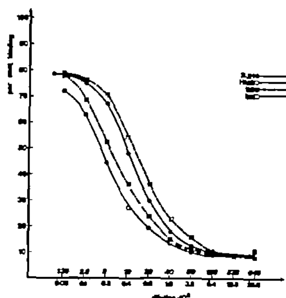


Fig 2 Titration of four antisera, RJH 19044 HMB, ES and BH against a dose of radiolabelled HBAg of about 6,000 cpm. Dilutions of RJH 19044 indicated above the abscissa, and of the other three sera below it.

peak. The active fractions were pooled and diluted so as to contain about 6000 cpm in 10 µl, i.e. the antigen dose used in each tube.

The four antisera, RJH 19044 HMB, ES and BH, bound approximately 80 per cent of the added radioactivity The sera were titrated by dilution in a pool of at least 10 negative human sera the results are presented in Fig. 2. All sera gave similar curves, *RJH 19044* reacting 25 to 50 times stronger than the other sera for which reason it was mainly used in the routine tests. For antigen testing, RJH 19044 was used in a dilution of 1/5000 BH was used in a dilution of 1/200 and the sera *HMB* and *ES* in a dilution of 1/100 These dilutions gave 60–70 per cent binding of the radioactivity against a back ground binding of approximately 10 per cent.

The time-curves of radioactive binding by the four antisera are presented in Fig 3 Maximum binding of all sera in the chosen dilutions was obtained after 60 minutes. Close to maximum binding was achieved after 30 minutes. No more radioactivity was found in the sediment after incubation for four days at 4° C. The time-curves applying to all four antisera were similar For antibody testing an incubation time of 1 hour at 37 C was chosen.

Antigen competition. 10 µl antigen dose (6000 cpm) 25 µl antiserum dilution, and normal serum or antigen containing serum in two different dilutions, 1/1 or 1/5 in normal serum, respectively were added to the system. The time-curves applying to the simultaneous mixing of the components are presented in Fig 4 The kinetics of the reaction is similar whether or not cold antigen is present, and maximum binding of each of the reactions was obtained after one hour of incubation at 37° C. If cold antigen was present *in a dilution 1/5 maximum binding* would be about 55 per cent, and if present undiluted, the maximum binding would be 35 per cent.

To secure that the specificity of the antigen was preserved after the labelling process, the purified preparation of HBAg was added to the system and was found to inhibit completely the binding capacity of the four antisera. (In the figure, 100 per cent inhibition corresponds to the back-ground radioactivity in the sediment.) This is shown in Fig. 5 where the preparation was used in different

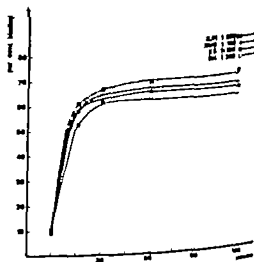


Fig 3 Effect of time on the reaction between radiolabelled HBAg (6,000 cpm) and four different antisera diluted as indicated at 37 C.

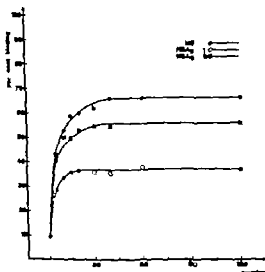


Fig 4 Time curves for simultaneous mixing of radiolabelled HBAg (6,000 cpm) antiserum RJH 19044 1/5000 and two different dilutions of an HBAg positive serum in a normal human serum pool. The serum pool was used as control (NS)

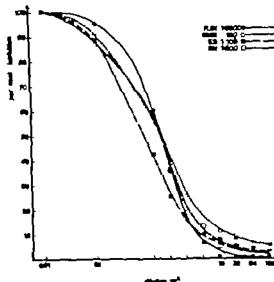


Fig 6. Titration of an HBAg positive serum, using four different antisera at indicated dilutions and a radiolabelled antigen dose of 6,000 cpm. All dilutions of the antigen containing serum were made in a pool of normal human sera.

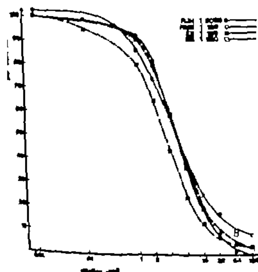


Fig. 5 Titration of the HBAg preparation before radiolabelling, using four different antisera at indicated dilutions and a radiolabelled antigen dose of 6,000 cpm. All dilutions of the HBAg were made in pool of normal human sera.

dilutions in a negative human serum pool. Fifty percent inhibition was obtained, using the same dilution between 1/4000 and 1/8000 of the preparation, for three of the antisera, RJH 19044 HMB, and BH, while this was obtained in the case of antiserum ES by a dilution below 1/4000.

The serum from a patient who had been using injections of narcotics and who came to be a persistent carrier of HBAg after clinical hepatitis, was titrated in the system using the four different antisera. The result is presented in Fig. 6. Even in this case, 50 per cent inhibition was obtained with the same dilution of antigen for the three antisera RJH 19044 HMB, and BH, while a somewh... lower dilution was necessary for the anti... ES The curves in Fig. 5 and Fig. 6 ... that antiserum ES behaves somewhat ... ently than the others. The reason ... present not known, but the assum... it has to do with antigenic subty... investigation.

Sensitivity and specificity 1 ... sera that were negative in th... roelectrophoresis test ...

TABLE 1 *Results of Staphylococcal RIA Test on Panel W-C of 1973 HBAg Proficiency Evaluation Programme**

Sample No.	Correct answer All methods	Correct answer RIA	Staphylococcal RIA	Agreement
41	–	–	–	Yes
42	–	+	10	Yes
43	+	+	100	Yes
44	–	–	– (10)†	Yes
45	–	+NC‡	–	No
46	–	+NC	1 (1)	Yes
47	+NC	+	100 (1)	Yes
48	+NC	+	100	Yes
49	–	–	– (10)	Yes
50	+	+	100	Yes
51	+	+	100	Yes
52	–	+	10	Yes
53	+NC	+	100 (1)	Yes
54	+	+	100	Yes
55	+	+	100 (1)	Yes
56	–	–	–	Yes
57	–	+	1 (1)	Yes
58	–	–	–	Yes
59	+NC	+	10	Yes
60	–	–	– (10000)	Yes

* Sponsored by College of American Pathologists and American Association of Blood Banks.
‡ Non concensus, 75 per cent agreement between referee laboratories.
† Numbers in brackets indicate antibody titres.

Dr *A M Prince** The New York Blood Center and were studied by the staphylococcal test. The sera had been tested by the Ausria test for HBAg and by the passive hemagglutination test for HBAb. There was agreement in 19 out of 20 sera. One serum was in both laboratories found to contain both HBAg and HBAb. There was disagreement in the case of one serum which was found to contain a very weak HBAg using the Ausria test and HBAb if the staphylococcal test was used. 2. Twenty coded sera belonging to panel W-C of the 1973 HBAg proficiency evaluation program sponsored by the College of American Pathologists and the American Association of Blood Banks were tested for both HBAg and HBAb the results are presented in Table 1 There was agreement in 19 out of 20 results. Serum 45 gave negative results by the staphylococcal test on repeated trials, but it was considered positive in 75 per cent of the referee laboratories using RIA tests. Serum 46 gave also non concensus results in the referee laboratories, and as serum 45 this serum was considered positive by 75 per cent of them. Samples 46 and 57 were duplicates and both were positive if the staphylococcal test was used. Samples 42, 48, 52, 55 and 59 were different dilutions of one and the same serum (1/32, 1/8, 1/32, 1/1 and 1/8) and gave the titres 10 (< 100), 100 (< 1000) 10 (< 100) 100 (< 1000), and 10 (< 100) using the staphylococcal test. Samples 50, 51 and 54 were triplicates of the same serum. The titres obtained with the decimal dilution steps in the staphylococcal test compare well with the original dilutions and the results obtained on the duplicates and triplicates, respectively were identical. Antibody titres obtained with the staphylococcal test are listed in brackets in the table

* We are indepted to Dr Prince for the supply of the coded sera.

the antibody results on different sera. According to our experience with the test where controls with known infections have been available, the use of 2 standard deviations from the mean seems to give specific results according to statistical theory

In practice, screening of sera is performed with a 3 per cent suspension in order to economize with the staphylococci. This corresponds to the testing of approximately 400 tubes per litre of bacterial growth medium. Large concentrations of IgG in a serum may however exceed the IgG-binding capacity of a 3 per cent suspension and thus simulate a low titre result of HBAg Retesting of the serum with a 10 per cent staphylococcal suspension has always eliminated such a depression of the radioactive counts unless clear indications of a hepatitis infection are present. A further testing of groups of patients with chronic hepatitis and hypergammaglobulinaemia may throw more light on this problem.

The excellent technical assistance of *Oddveig Flatåker* and *Marit Nielsen* is gratefully acknowledged.

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ANTIBODIES WITH RESTRICTED ELECTROPHORETIC HETEROGENEITY IN RABBITS HYPERIMMUNIZED WITH *KLEBSIELLA PNEUMONIAE*

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After hyperimmunization with *Klebsiella pneumoniae* type 2, type 3 or both, randomly bred rabbits would in most cases produce antibodies with restricted heterogeneity directed against the type specific polysaccharide. The simultaneous use of two antigens did not appear to alter the response to either antigen.

In the last few years, a number of reports have dealt with the fact that rabbits hyperimmunized with streptococcal or pneumococcal polysaccharides very often produced antibodies with restricted heterogeneity. *Osterland et al.* (12) reported in 1966 that intravenous immunization of rabbits, using streptococcal vaccines, elicited antibodies with restricted range of electrophoretic properties compared with normal γ-globulin. The streptococcal antigens were relatively simple polysaccharide polymers.

The same effect was obtained with another bacterial vaccine. *Haber* (5) immunized one group of rabbits with type III and a similar group with type VIII pneumococci. In the majority of animals he found an increase in the content of γ-globulin, but it was only in a small group that a marked increase in quantity *and* a restriction in electrophoretic mobility could be demonstrated. *Haber* suggested that the restricted heterogeneity could be related to one single cell clone outgrowing other clones capable of responding to the same antigen. The capacity for efficient growth could be a heritable property.

Further studies of the genetic control of the immune response to streptococcal carbohydrates were published in 1971 by *Eichmann* & *co-workers* (2). They claimed that the amount of antibodies produced after immunization as well as the degree of restriction of the heterogeneity of the antibodies were under genetic control. But, in addition to the genetic factor, the properties of the antigen seemed to be of importance, e.g. the number of immunodominant groups and the regularity of their attachment to the carrier.

This hypothesis was not supported by the work of *Kimball et al.* in 1971 (9). They reported that most, if not all, rabbits will produce antibodies with restricted electrophoretic mobility to pneumococcus type III polysaccharide. They also deduced that a critical factor in the response appeared to be the repeated antigenic stimulus rather than the genetic constitution of the animals.

Another observation by the same authors

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was that two of the rabbits which produced high quantities of antibodies with restricted heterogeneity also showed clinical signs similar to those associated with multiple myeloma in man.

This was contrary to *Haber's* report in 1970 (5). He suggested that, in spite of the fact that one cell clone took over the production of antibodies, it did not represent a malignant transformation since antibody production remained under the control of antigen stimulation.

In a work by *Kimball* published in 1972 (10) the affinity of the rabbit antibodies directed against the capsular polysaccharide of type III pneumococci was examined at various times during the immunization. By means of equilibrium dialysis he found that every rabbit produced antibodies of increasing affinity during most of the time they were continuously immunized. But this did not go on indefinitely. In the latter part of the response, a marked decrease in the affinity of the serum antibodies appeared, accompanied by a decrease in concentration. He concluded that restricted antibody components with high affinity represented products of cell clones with great efficiency of trapping antigen, in competition with antigen sensitive cells of lower affinity and with serum antibody. He also assumed that the productive phase of a given clone was limited. After immunization for a long time there were no longer any antigen sensitive cells with receptors of an affinity higher than that of the serum antibody and a decrease in affinity and quantity would appear.

The development of the immune response to type III and VIII pneumococcal polysaccharides was studied by *Chen et al.* (1) in 1973. They found that 67 out of 70 rabbits at one time or another produced antibodies of restricted electrophoretic mobility but that no general pattern could be defined.

The main purpose of the work reported here was to examine whether the capsular polysaccharide of *Klebsiella pneumoniae* type 2 and type 3 also induced antibodies of restricted heterogeneity in rabbits.

Another matter of interest was to see what happened if two different polysaccharide antigens were used at the same time.

MATERIALS AND METHODS

Antigen. *Klebsiella pneumoniae* type 2, strain F.24 and *Klebsiella pneumoniae* type 3, strain F.10.K.Y were grown in broth for 18 h. The culture was used as antigen for immunization.

Polysaccharides. Capsular polysaccharides from the two *Klebsiella* types mentioned above were isolated as described earlier (3) (13). The polysaccharide was used as antigen in quantitative precipitations and in haemagglutination.

Immunization. Randomly bred rabbits were used for immunization. Fifteen rabbits were divided into three groups. Rabbits nos. 1 to 5, (group 1) were immunized with *Klebsiella pneumoniae* type 2, strain F.24. Rabbits nos. 6 to 10, (group 2) received *Klebsiella pneumoniae* type 3, strain F.10.K.Y. Rabbits nos. 11 to 15 (group 3) were immunized both with type 2 and type 3. In this group, one rabbit (no. 11) died after only three injections and the group therefore contained only four rabbits throughout the rest of the period.

All fourteen rabbits were immunized intravenously in one and the same way. For the first three injections, the bacteria were killed by formalin. The amounts were 0.1, 0.2 and 0.3 ml. The subsequent immunizations were given as 0.3 ml of living broth culture. The third group of rabbits received 0.3 ml consisting of equal amounts of both *Klebsiella* types. The rabbits were immunized once a week for four months. During the next six or seven months the immunizations were performed twice a week. Blood was collected from the ear every second week, and by heart puncture once a month. Each sample was numbered in succession as the sera were collected.

Electrophoresis. All blood samples were examined by electrophoresis on cellulose acetate. The electrophoresis was run in a *Gelman* electrophoresis chamber on cellulose acetate strips, (25 x 170 mm) in Gelman high resolution buffer at pH = 8.8, and ionic strength = 0.06. The electrophoresis was run

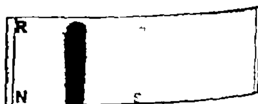


Fig. 1. Electrophoresis of serum from a rabbit before the immunization started. *s* indicates the start line.

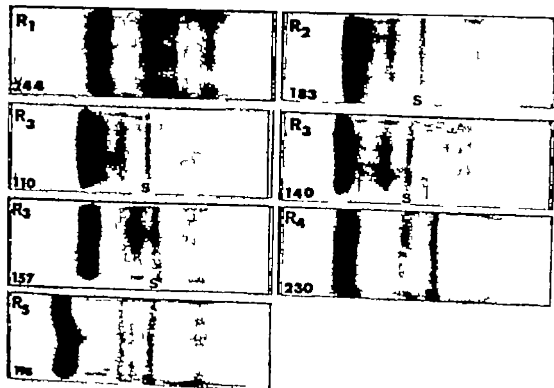


Fig. 2 Electrophoreses of serum from rabbits immunized with *Klebsiella pneumoniae* type 2, strain F.24 (Table 1)

for 1 h at a constant voltage of 450 V The current was 2–3 mA per strip, or approx. 1 mA/cm.

After 1 h, the strips were put directly into Ponceau dye for 10 min. Excess of background stain was removed by washin the strips in 5 per cent acetic acid. The strips were dried in the air before they were clarified.

The dried strips were first put in a freshly made solution of 10 per cent acetic acid in ethanol for 30–60 sec. They were placed on a microscope slide (26 × 76 mm) pressed flat and the ends were cut off. After few minutes, the slides were dipped quickly in a solution of 30 per cent ethylacetate in acetic acid. After drying the slides were ready for examination.

Quantitative determination of antibodies against type specific polysaccharide was carried out by precipitation, using the Biuret method (7) for determination of protein.

When the rabbits received two antigens simultaneously quantitative precipitations were carried out with both polysaccharide antigens. For absorption, the quantity of polysaccharide giving the maximum precipitate, was used. After addition of one polysaccharide, the supernatant containing antibodies against the other type was examined by electrophoresis on cellulose acetate.

Passive haemagglutination It applies to all sera that the content of antibody was determined by passiv haemagglutination (4)

RESULTS

Before any immunization started, blood samples were collected from all rabbits and examined by electrophoresis on cellulose acetate as zero value. Fig 1 is a picture of one of the sera drawn before the immunization started. This picture is representative of the zero-samples from all rabbits.

The antibody response was determined in all sera by electrophoresis on cellulose acetate and by passive haemagglutination. Quantitative determination of specific antibody against the type specific polysaccharide was carried out only in some of the sera.

The rabbits in group 1 received between 23 and 48 injections in the course of 140 to 249 days. The results are reported in Table 1 and Fig 2. The figures in the table originate

TABLE 1 *Antibody Response to* Klebsiella pneumoniae *Type 2 Strain F.24 in Hyperimmunized Rabbits*

Rabbit no. and serum no.		No. of injections and days after start of immuniz.		Haemagglutination titre*	Mg antibody per ml serum§	Antibody of restricted heterogeneity	Comment
1	62	9	62	1024		–	
1	93	12	82	256	6.6	–	
1	171	31	166	256		–	Marked γ-globulin region.
1	184	35	180	256		+	Restricted heterogeneity, two bands which keep through the immuniz. period.
1	220	45	222	256	16.9	+	
1	244	48	249	1024	16.7	+	Photo in Fig. 2.
2.	49	6	47	512		–	
2.	139	19	129	1024	5.2	–	
2.	172	30	166	1024		–	Well marked γ-globulin region.
2	183	33	174	1024	7.5	+	One band. Photo in Fig 2.
3.	50	6	47	256		–	Well marked γ-globulin region.
3	110	15	110	512		+	Restricted heterogeneity one band. Photo in Fig. 2.
3	140	19	129	512	21.8	+	Two bands. Photo in Fig 2.
3	146	22	138	512		+	Two bands, but not so strong any longer
3.	157	23	140	1024	13.7	+	Restricted heterogeneity Photo in Fig. 2.
4	31	4	31	2048	3.0	–	
4	173	31	166	256		+	Restricted heterogeneity one weak band.
4	209	42	215	512		+	Restricted heterogeneity one band
4	230	45	232	512	20.6	+	Restricted heterogeneity two bands. Photo in Fig. 2.
5.	38	4	34	1024		–	
5.	52	6	47	1024		–	
	123	16	117	1024	8.1	–	
	195	36	185	1024	6.2	+	Restricted heterogeneity Photo in Fig 2.

* Reciprocal of highest dilution giving distinct haemagglutination.
§ When no numbers given, quantitative analysis is not done.

from a large number of data. The results in Table 1 indicate that all five rabbits in this group produced antibodies with restricted heterogeneity In four of the rabbits immunization had to be repeated more than 30 times before they produced antibodies with restricted heterogeneity One of the rabbits (no. 3) showed restriction after 15 injections.

Three of the rabbits (no. 1 3 and 4) produced *two different antibody fractions* with restricted heterogeneity against one and the same antigen. This is demonstrated in the photos of serum 244 140 and 230 in Fig. 2 where two distinct bands can be observed in the γ-globulin region.

The rabbits in group 2 received between 37 and 63 immunizations in the course of 187 to 378 days. The results of the examination is reported in Table 2 and Fig 3. In this group two of the rabbits *did not respond* with antibodies of restricted heterogeneity (rabbit no. 8 and no. 10) Sera from both rabbits showed low quantities of specific antibodies against polysaccharide type 3, if ex-

TABLE 2. *Antibody Response t* Klebsiella pneumoniae *Type 3 St ain F 10 N Y in Hyperimmunized R bbits*

Rabbit no. and serum no.		No. of injections and days after start of immuniz.		Haemagglutination titre	Mg antibody per ml serum§	Antibody of restricted heterogeneity	Comment
6.	39	5	34	256		+	Restricted heterogeneity one band can easily be seen. Photo in Fig. 3
6.	53	7	47	512		–	No restricted heterogeneity
6.	67	9	61	1024		–	
6.	196	37	187	256	0.7	–	
7	54	7	47	256		–	
7	142	20	129	1024	5.0	–	
7	188	35	180	1024		+	Restricted heterogeneity one narrow band.
7	238	45	222	5120	31.2	+	One strong band. Photo in Fig. 3
7	251	57	271	5120	25.0	+	One band. Photo in Fig 3
7	261	63	326		15.0	+	Restricted heterogeneity but not so strong as before.
8.	25	3	20	512		–	No restricted heterogeneity
8.	165	27	152	256	2	–	
8.	223	45	222	512	1 6	–	
8.	247	53	257	1024	_7	–	Photo in Fig. 3
8.	62	63	328	256	0 9	–	
9	42	5	34	1024		+	Restricted heterogeneity Photo in Fig. 3
9	91	12	81	2048	5 9	–	Marked γ-globulin region.
9	166	27	152	1024		+	Restricted heterogeneity Photo in Fig 3
9	178	31	166	1024	12 5	+	Restricted heterogeneity One band is easily seen.
9	224	45	222	1024	17 1	+	Restricted heterogeneity
9	253	57	271	4096	13 7	+	Restricted heterogeneity
9	263	63	326	256	_5	–	No restricted heterogeneity any longer Photo in Fig 3
10	25	3	20	256		–	
10.	167	27	152	256	1 6	–	
10	225	45	222	256	2 6	–	
10	234	57	271	256	3 1	–	Phot in Fig. 3
10	264	61	326	256	1.2	–	

Reciprocal of highest dil tion giving distinct haemagglutination.
§ When no numbers given, quantitative analysis is not done

amined by precipitation. The same applies to rabbit no. 6, but this rabbit produced antibodies with restricted heterogeneity already after five injections. No quantitative determination of antibody in this sample was carried out, but the haemagglutination titre was low

The rabbits in group 3 received between 16 and 61 immunizations in the course of 112 to 284 days. The antibody response in this group was examined in the same way as in the other groups the results are shown in Table 3 and Fig. 4

One rabbit, no. 15 produced specific antibodies against the capsular polysaccharide, but not with restricted heterogeneity The animal was in a poor state and was killed after 16 immunizations.



Fig 3 Electrophoreses of serum from rabbits immunized with *Klebsiella pneumoniae* type 3 strain F 10,NY (Table 2)

The other three rabbits in this group produced antibodies with restricted heterogeneity The antibodies seemed to be against both polysaccharide antigens. Rabbit no. 12 and no. 14 produced approximately the same quantity of antibodies against both of the type specific polysaccharides. Rabbit no. 15 on the other hand, produced much more antibodies against *Klebsiella* type 2 than against type 3

Absorption of serum with one polysaccharide, and new electrophoresis of the supernatant, showed that antibodies against the other polysaccharide were left.

Absorption carried out with serum 204 from rabbit no. 14 showed that the strong and narrow band represented antibodies against *Klebsiella* type 2, while the wider and weaker band contained antibodies against type 3 in spite of the fact that antibodies against the two types were present in the same quantity

As regards rabbit no. 15 serum 245 was absorbed with the two type specific polysaccharides, one at a time Antibodies against both types were represented by fractions with restricted heterogeneity even though antibodies against type 3 only were present in small quantities.

DISCUSSION

Rabbits hyperimmunized with *Klebsiella pneumoniae* produced antibodies with restricted heterogeneity against the capsular polysaccharide This is in agreement with earlier reports, according to which streptococcus and pneumococcus had been used as antigens. In all cases, antibodies of molecular uniformity and specificity against the capsular polysaccharide were produced.

The amount of antigen and the frequency of immunizations can be different, but all injections were given intravenously It applies

TABLE 3 *Antibody Response to* Klebsiella pneumoniae *Type 2 strain F.24* and *Type 3 Strain F.10.N.Y in Hyperimmunised Rabbits*

Rabbit no. and serum no.	No. of injections and days after start of immuniz.		Haemagglutination titre* ps. 2	ps. 3	Mg antibody per ml serum§ ps. 2	ps. 3	Antibody of restricted heterogeneity	Comment
72	9	61	4096	?048			+	One band in the γ-globulin region. Photo in Fig. 4
125	17	117	2048	1024	23 7	11.2	+	Restricted heterogeneity one band.
203	38	200	4096	2048			+	Restricted heterogeneity two bands. Photo in Fig. 4
219	43	220	1024	2048	19 4	15.7	+	The two bands are weaker.
28	3	20	2048	512			–	
45	5	34	4096	512			–	
12?	16	112	?048	1024			–	Photo in Fig. 4
92	12	81	800	800	8.1	17.5	–	The γ-globulin region is well marked. Photo in Fig. 4
120	16	110	800	800			+	Restricted heterogeneity one band. Photo in Fig. 4
169	27	152	3200	1600	38 7	33 1	+	Restricted heterogeneity
193	33	180	3200	3200	68.7	35.6	+	
204	38	200	3200	3200	62.0	58.5	+	One very strong and narrow band and a wider band, perhaps two. Photo in Fig. 4 The narrow band is removed by abs. with ps. 2 but not with ps 3. Photo 204a and 204b in Fig. 4
233	58	271	3200	3200	48.7	49.3	+	Photo in Fig 4.
236	61	284	6400	6400	51.2	43.6	+	Very strong restricted heterogeneity
30	3	20	800	100			+	Restricted heterogeneity
47	5	34	1600	800			+	Photo in Fig. 4
138	18	123	1600	800			+	
143	20	129	1600	800	9.6	4 4	+	
170	27	152	1600	800			+	
203	38	200	6400	400			+	Two bands. Photo in Fig. 4
–	45	222			36.2	4 4	+	
245	32	256	6400	400	37.5	5 0	+	One strong and one weak band. Photo in Fig. 4 Absorption with ps. 2, but not with ps. 3 removed the strong band. Photo 245a and 245b in Fig. 4

* Reciprocal of highest dilution giving distinct haemagglutination

ps. = Capsular polysaccharide.

§ When no numbers given, quantitative analysis is not done

to the streptococcus and the pneumococcus that the bacteria were heat-killed by formalin. In this work, the immunization schedule was introduced by administration of a formalin-killed broth culture of the *Klebsiella* to the rabbits. After three immunizations, the rest was given as a living culture. Eleven rabbits out of fourteen produced antibodies with restricted heterogeneity against *Klebsiella* polysaccharide. Only randomly bred rabbits were

of cells can very well produce antibodies with restricted heterogeneity against the same antigen. (Rabbit no 1 serum no. 244 rabbit no. 3 serum no. 140 and rabbit no. 4 serum no. 230 in Fig 2)

Some rabbits produce antibodies with restricted mobility early in the immunization period in contrast to other rabbits in which the production commences much later at least in quantities to be demonstrated by the methods used here. Two rabbits (no. 6 and no. 15) produced antibodies with restricted heterogeneity very early In the case of rabbit no 6 the fraction was demonstrated after 5 immunizations, but it disappeared again after further injections. Rabbit no. 15 produced antibodies with restricted heterogeneity already after 3 injections. This antibody fraction seemed to remain during further immunizations, and was still present after 27 injections.

If two polysaccharide antigens were used at the same time competition between the antigens was not apparent, if it is possible at all to draw conclusion from results obtained by examinations of three rabbits. One rabbit (no. 13) in this group did not at all form antibodies with restricted heterogeneity The other three rabbits produced restricted antibodies against both antigens.

So far there seemed to be no connection between the size of the total antigen dose and the ability to form antibodies with restricted mobility Continued antigenic stimulus seemed to stimulate established cell clones as well *as create new ones. When immunization* is carried out over many months, the pattern of restriction as well as the antibody concentration undergoes continual changes. When antibodies against the capsular polysaccharide are present in large quantities, restricted heterogeneity can mostly be demonstrated.

The production of antibodies with restricted heterogeneity by hyperimmunized rabbits most probably is determined by more than one factor But it seems obvious that the antigen structure must be important and in this connection, especially the polysaccharide part of the antigen. The capsular polysaccharides of *Klebsiella* type 2 and type 3 are relatively simple compounds. They have few antigenic determinants compared with a protein antigen, due to the fact that the structure consists of repeating oligosaccharide units. Some of the determinants were able to stimulate only few clones of antibody-producing cells.

This could explain the fact that both monoclonal and heterogenous antibodies against the same antigen are produced simultaneously A heterogenous population of antibody-producing cells is first stimulated by all determinants present. Most of the cell clones, however are later repressed to be overgrown by clones producing antibodies against the most dominant determinants, and the result will be antibodies with restricted heterogeneity

It has also been reported that rabbit antibodies, against artifical hapten-protein conjugate such as DNP pneumococci, DNP Insulin, or azo-p-benzoate bovine γ-globulin were of restricted heterogeneity (11) (8) (14) In this case the amount of antibodies was small.

It is not only in rabbits, but also in guinea pigs that antibodies with restricted heterogeneity are produced when 2 4 DNP-protein conjugate is used as antigen (6) Results obtained in this laboratory by immunization of randomly bred guinea pigs with *Klebsiella pneumoniae* type 3 showed that the antibody produced often was of restricted heterogeneity In this case the guinea pigs were immunized subcutaneously with a mixture of broth culture and Freund's adjuvant. The production of antibodies against the type specific polysaccharide was examined by haemagglutination, and by electrophoresis on cellulose acetate.

These results obtained in cases of rabbits immunized with simple hapten-protein conjugates, and guinea pigs immunized with hapten protein conjugates, or polysaccharide antigen, support the idea that the structure of the antigen must be of importance for a

production of restricted antibodies, beside other unknown factors.

In order to obtain general information and for examination of immune globulins, the production of antibodies with restricted heterogeneity against a known antigen, can be of great value. The antibodies may be isolated in quantities sufficient for structural work.

We wish to thank Mrs. *And Karin Standal* for skilled technical assistance, and Mr *Per Torgersen* for valuable help during the immunization procedure.

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ACQUIRED DEFECT IN THE BACTERICIDAL FUNCTION OF NEUTROPHIL GRANULOCYTES DURING BACTERIAL INFECTIONS

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The ingestion and killing of *Staphylococcus aureus* by peripheral blood leucocytes from 130 patients with infectious and non-infectious diseases have been determined, using an improved technique for the evaluation of neutrophil granulocyte function *in vitro*. A reversible defect in intraleucocytic killing could be demonstrated in granulocytes from patients with severe bacterial infections whereas granulocytes from patients with viral infections and non-infectious diseases showed only minor deviations from normal function by this technique. This defect could furthermore be attributed to malfunction of myeloperoxidase-mediated bactericidal systems and was found to be positively related to the degree of "shift to the left" of circulating myeloid cells. A similar defect could also be demonstrated in some patients with non-infectious granulocytosis. It is suggested that this defect may be caused by malfunction of a subpopulation of immature myeloid cells and/or by altered granulocyte function caused by *in vivo* activation of the cells due to immune reactions.

Phagocytosis by neutrophil granulocytes is of primary importance for the normal host defence against invading micro-organisms. Cooperation with antibodies and complement components enhances ingestion and renders the organisms susceptible to powerful intraleucocytic killing systems. With the description in 1966 of an intrinsic neutrophil defect in intraleucocytic killing associated with recurrent infections, chronic granulomatous disease (C.G.D.), attention was directed towards evaluation of those parts of cellular activity which lie beyond the stages of opsonization and ingestion (6). Since then, several rare primary disease syndromes characterized by defects in intraleucocytic killing have been recognized (3 5 14 23) but the activity of this function in acquired clinical disorders is much less explored. Temporary decrease in intraleucocytic killing in patients with severe burns developing sepsis (1) in some lymphoproliferative disorders (24) and in a patient with a mixed cryoglobulin (4) has been described. More recently *Solberg & Hellum* (25) have reported decreased staphylocidal activity of granulocytes from patients with bacterial infections. These findings are substantiated by the present studies which demonstrate an acquired defect in the staphylocidal activity of circulating phagocytic cells from patients with severe bacterial infections. This defect appears to be secondary to the

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infectious state and positively related to the degree of immaturity of circulating myeloid cells. Additional studies are presented which indicate that the decreased staphylocidal function can be attributed to malfunction of myeloperoxidase-mediated systems.

MATERIALS AND METHODS

The method employed has been described in detail in a previous publication (12) 2.5×10^6 per ml polymorphonuclear leucocytes from dextran-sedimented blood were mixed in an approximately 1 1 ratio with *St phylococcus aureus* in gelatinized, heparinized Hank's balanced salt sol tion in the presence of 10 per cent pooled normal human serum. For some initial experiments *Staph au eus* phage type 42 E+ was employed, but most experiments were done with the more widely used strain 502 A and no difference in the behaviour of granulocytes towards the two organisms could be detected. Incubation mixtures of 2.0 ml were set up in duplicate at 35 C with end over end rotation at 20 rev/min. To one set of tubes, penicillin, 100 iu per ml, and streptomycin, 100 μg per ml were added after 15 minutes incubation to inactivate non-ingested bacteria. Samples were removed at 2 and 4 or 1½ and 3 hours incubation. Total survi ing colony-forming units (C.F U) *Staph. aureus* were determined from tubes without antibiotics *after lysis of the cells in distilled* water serial dilution, and pour-plating by conventional technique Intracellular C.F U were determined from tubes with antibiotics after washing of the cells, lysis, serial dilution, and pour-plating In some experiments a third tube was set up, identical to the one with antibiotics except that sodium azide 1×10^{-3} M was added at the start of the incubation to inhibit ntraleucocytic killing (13)

This method has been extensively analysed in previous publications (12 13) which allowed the following conclusions to be drawn. The method is particularly sensitive in revealing decreased intraleucocytic killing which causes an increase intra cellular C.F U By normal granulocyte function this cannot be caused by increased ingestion since this leads to a proportional increase n ntraleucocytic killing (12) When intraleucocytic killing is markedly reduced, in C G D an increase in total C.F U will also be seen Defects in ingestion will cause an increase in total C.F U with nearly unchanged intracellular C F U Such defects are however not always disclosed by thi type of technique since the rotation system circumpasses mobility and chemota is of the leucocytes

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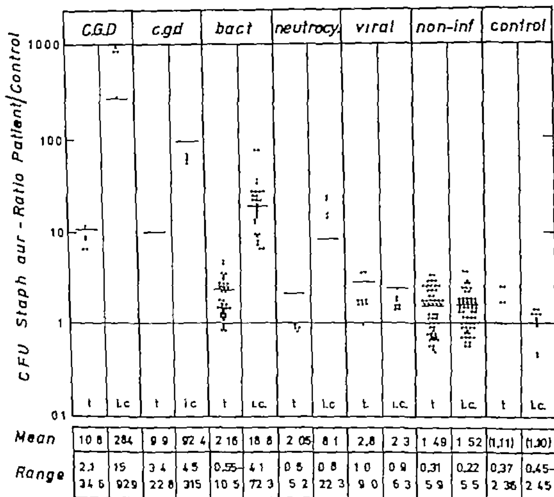
Patients The present studies describe the results obtained in 130 patients who have been classified into the following 4 groups: 1) 44 patients with bacterial infections, notably meningitis, lower respiratory tract infections, and bacteraemia—all with a positive microbiological diagnosis. 2) 13 patients with viral infections without complicating bacterial infection. 3) 12 patients with non-infectious granulocytosis, defined as more than 10,000 circulating myeloid cells, notably acute myocardial infarction. 4) 61 patients with a variety of non-infectious diseases, notably rheumatic, neoplastic, and cardiopulmonary diseases among whom several were running a temperature at the time of testing The group includes 24 patients who had recovered from infectious diseases the study of these formed part of a screening program by which to obtain evidence of decreased resistance to infection, but all of the patients were without signs of infection for at least 10 days prior to testing. Included are also the results obtained in 7 patients with C.G.D. and in 7 heterozygous carriers of C G.D together with 14 paired determinations in normal persons.

At the beginning of these studies, attention was not primarily focused on a possible relationship of granulocyte function t granulocytosis (vide infra) and consequently reliable leucocyte count from the same day as that on which testing was carried out was not a ailable in all patients. The results obtained on the day of testing were as follows 1) Bacterial infections a leucocyte count was obtained in 34 of 44 patients and 22 had more than 10,000 myeloid cells per μl blood. 2) Viral infections leucocyte count were obtained in 7 of 13 patients and all had less than 10,000 myeloid cells. 3) Non-infectious gran locytosis all 12 patients had more than 10,000 myeloid cells. 4) Non-infectious diseases leucocyte counts were available in 22 of 61 patients all of whom had less than 10 000 myeloid cells per μl blood. For mean values and range of mature and immature myeloid cells in groups 1) and 3) see also Table 1

RESULTS

The results of all individual determinations in the different patient groups are graphically depicted in *Fig 1 in a logarithmic scale* The function of granulocytes from patients with

These results have been reported previously (11)



	C.G.D t	C.G.D i.c.	c.gd t	c.gd i.c.	bact t	bact i.c.	neutrocy. t	neutrocy. i.c.	viral t	viral i.c.	non-inf t	non-inf i.c.	control t	control i.c.
Mean	10 8	284	9 9	92 4	2 16	18 8	2 05	8 1	2.8	2 3	1 49	1 52	(1.11)	(1.10)
Range	2.1–34 6	15–929	3 4–22 8	4 5–315	0.55–10 5	4 1–72 3	0 8–5 2	0 8–22 3	1 0–9 0	0 9–6 3	0,31–5 9	0.22–5 5	0,37–2 36	0.45–2 45

Fig 1 Total (t.) and intracellular (i.c.) colony-forming units (C.F.U.) *Staph. aureus* using peripheral blood leucocytes from 1) 7 patients with chronic granulomatous disease (*C.G.D.*)–(13 determinations) 2) 7 heterozygous carriers of C.G.D. (*g.d.*) 3) 44 patients with bacterial infections (*bact.*) 4) 12 patients with non-infectious granulocytosis (> 10,000 myeloid cells per μl blood) (*neutrocy.*) 5) 13 patients with viral infections (*viral*) 6) 61 patients with non-infectious diseases (*non-infect.*) 7) 14 healthy normal adults (*control*). Results are expressed as the ratio of patient values over simultaneously determined values in a normal control. Note logarithmic scale. For explanation of results marked with triangles—see text.

bacterial infections differs markedly from normal function. The reduction in total C.F.U. is significantly decreased compared to the normal control granulocytes run simultaneously ($p < 0.001$ Mann-Whitney rank sum test). This is clearly due to defective intraleucocytic killing as evidenced by the pronounced increase in intracellular C.F.U. Intracellular C.F.U. in granulocytes from patients with bacterial infections is furthermore significantly higher than in granulocytes from the group of non-infectious diseases ($p < 0.001$ Mann-Whitney rank sum test). This large heterogeneous group displays only minor deviations from normal function by this technique and the distribution of individual results corresponds roughly to the distribution of paired determinations in normal persons.

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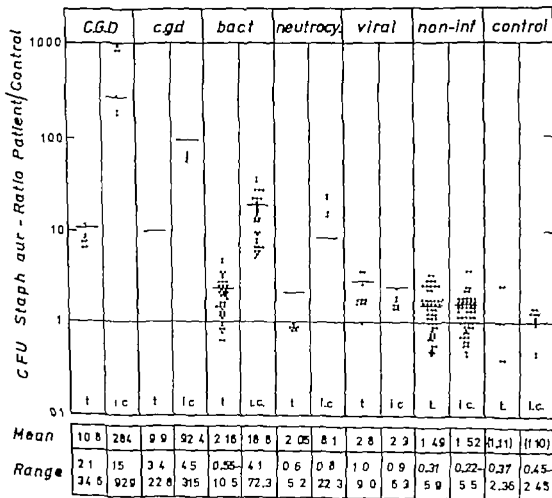
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TABLE 1 *Total Myeloid and Percentage of Immature (Band Forms or Younger) Myeloid Cells in Patients with Bacterial Infection and Patients with Non Infectious Granulocytosis at Time of Granulocyte Function Testing (Fig 1)*

	Number of patients	Leucocyte count done in:	Total myeloid cells per µl blood		Percentage immature myeloid cells	
			mean	range	mean	range
Bacterial infection	44	34	12,670	2,260–32,900	11.7	0–51.6
Non-infectious granulocytosis	12	12	12,410	10,000–17,390	3.5	0–12.5

is not as extreme as that in C.G.D patients and carriers of C.G.D It is evident from Fig 1, however that some overlapping between these groups does exist, but most of this is undoubtedly caused by day to day variations of the method.

In the group of viral infections, the increase in total C.F U is seen to be slightly higher than the increase in intracellular C.F U contrary to the other groups of patients. By the present technique, a decrease in the ingestion of the test-organism will cause an increase in total C.F U with nearly unchanged intracellular C.F U (12) and accordingly this finding could indicate decreased ingestion capacity of granulocytes from patients with viral infections.

In some patients, non-infectious granulocytosis appears to lead to a decrease in intra leucocytic killing similar to that seen in patients with bacterial infections, although the changes are not equally pronounced. As shown in Fig 3 the defective granulocyte function in patients with bacterial infections may in part be related to the degree of immaturity of circulating myeloid cells. The mean value and range of circulating myeloid cells and percentage of immature cells for these two groups are therefore given in Table 1 which shows that although the mean number of total myeloid cells is the same, the mean percentage of immature cells is much higher in patients with bacterial infections.

The group of bacterial infections includes 26 patients with bacterial meningitis. In three patients, marked with triangles in Fig 1 bacterial meningitis was suspected at the time of admission and all received treatment with high doses of penicillin, sulphonamides, and streptomycin, but all microbiological examinations were negative. In retrospect, the diagnosis seems possible only in one case In one case it turned out to be an intracranial aneurism, and in one a viral meningitis. In all of these the intracellular C.F U was only slightly increased (Fig 1)

The defective staphylocidal activity of granulocytes from patients with bacterial infections was further studied utilizing an improved technique based upon the blocking effect of sodium azide NaN_3, upon granulocyte function. NaN in a concentration of 1×10^{-4} M will cause nearly complete inhibition of staphylocidal activity without interfering with ingestion (13) Fig 2 shows the results of these studies in 8 patients with bacterial meningitis (13 determinations within 3 days of onset of disease) compared to normal controls studied simultaneously The ratio of intracellular C.F U between azide-treated and untreated cells is an expression of the activity of azide-sensitive bactericidal systems. In normal controls, the mean value of this ratio was 123 8 in the patients, this value was reduced to 10.4 A more than 10-fold decrease in azide-sensitive bactericidal activity could thus be demonstrated in these patients. Column A in Fig. 2 shows that slightly more intracellular C.F U could be recovered from azide-treated patient leucocytes than from azide-treated control leucocytes. This finding indicates that the ingestion by the patient leucocytes is at least as good, if not slightly increased compared to normal controls.

Since the results recorded in Fig 1 and

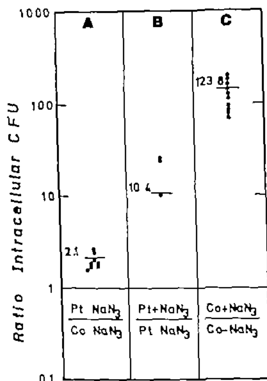


Fig 2 Effect of 1 × 10⁻³ M sodium azide (NaN) upon the granulocyte function of 8 patients with bacterial meningitis (13 determinations within 3 days of onset of disease) and of simultaneously determined normal controls. Column A ratios of intracellular colony-forming units (C.F U) *Staph. aureus* in NaN_3-treated patient leucocytes over NaN -treated control leucocytes. Column B ratios of intracellular C.F U in NaN -treated patient leucocytes over untreated patient leucocytes. Column C ratios of intracellular C.F U in NaN treated control leucocytes over untreated control leucocytes.

Table 1 in patients with bacterial infections and with non-infectious granulocytosis could indicate a relationship between granulocytosis and impaired granulocyte function, intra cellular C.F U was plotted against the proportion of immature myeloid cells in Fig. 3 The data include 22 patients with bacterial meningitis. Statistical calculations revealed a significantly positive correlation (Spearman's correlation coefficient R = 0 4794 $p < 0.05$) between these parameters.

Twelve of the patients with bacterial meningitis were re-tested one or more times during recovery Fig. 4 shows that all patients reverted to normal granulocyte function upon successful recovery from the infection. One patient (H.F) initially recovered from the meningitis but was left with serious impairment of cerebral functions and developed later a pneumonia and died. One patient (K.K.) was tested within two hours after admission and the staphylocidal activity of her granulocytes initially deteriorated, thereafter improving at about the same rate as that seen in the remaining patients. Normal granulocyte function was regained within approximately 2 weeks after institution of adequate therapy depending in part upon the degree of initial impairment.

DISCUSSION

The present studies demonstrate a decreased staphylocidal activity of peripheral neutrophil granulocytes from patients with severe bacterial infections, thus supporting recent obser

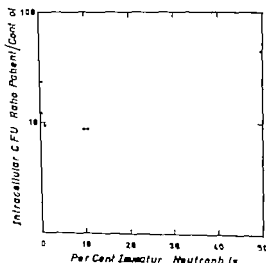


Fig 3 Relation of staphylocidal activity to degree of shift to the left" in 22 patients with bacterial meningitis I tracellular colony-forming units (C.F U) *Staph. aureus* expressed as the ratio of patient values over simultaneously determined control values are plotted against the percentage of immature myeloid cells (band forms, or younger) in the circulation. Statistical calculations showed positive correlation (Spearman correlation coefficient R: 0.4794 $p < 0.05$)

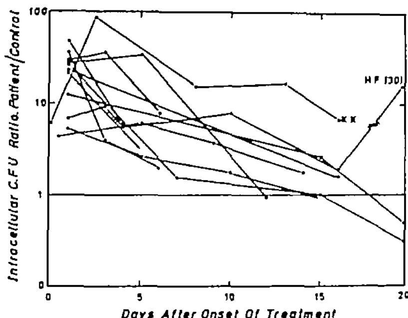


Fig 4 Relation of staphylocidal activity to duration of treatment in 12 patients with bacterial meningitis. Intracellular C.F.U expressed as in Figs. 1 and 3 Patients H.F and K.K.: see text.

vations by *Solberg & Hellum* (25) The experiments shown in Fig 2 moreover demonstrate that this defect can be ascribed to a decreased function of azide-sensitive bactericidal systems. Among several intraleucocytic bactericidal systems described, the one composed of hydrogen peroxide and granular myeloperoxidase possibly in conjunction with an oxidisable co-factor is likely to be of primary importance for the initial killing of a number of micro-organisms including *Staphylococcus aureus* (7 17 18) This system is extremely susceptible to blocking with sodium azide (9 13) probably caused by inhibition of myeloperoxidase (7) The data shown in Fig 2 indicate that ingestion is normal or slightly increased during bacterial infection, thus supporting recent observations by *Stossel* (26) Conversely slight impairment of azide-insensitive systems may also be present, but if so, by far less intense than the impairment of azide-sensitive systems (Fig 2)

The staphylocidal activity of granulocytes from patients with a large variety of non infectious diseases is much closer to normal function and this large heterogeneous group does not display the dramatic changes seen in granulocytes from patients with bacterial infections. In acute viral infections there may be slight impairment of ingestion of *Staph. aureus* but the present type of technique is not suitable for the detection of all ingestion defects since it circumpasses mobility and chemotaxis.

The changes in granulocyte function during bacterial infections seem to be the result from rather than the cause of the infectious state since 1) an initial deterioration of staphylocidal activity was seen in several patients during early stages of infection and 2) all patients followed, reverted to normal granulocyte function upon successful recovery (Fig 4)

In search of a cause of this defect, the possibility of an effect of the antimicrobial treatment, or other types of treatment, should be carefully considered. Many of the patients with bacterial meningitis received sulphonamides and hydrocortisone and both agents have been shown to inhibit the staphylocidal activity of normal neutrophils when added to the cells *in vitro* (15 20) The concentra-

tions necessary to demonstrate this effect were, however well above those normally achieved in the serum during treatment. Furthermore, improvement in granulocyte function could be demonstrated in many patients during actual treatment with these agents (Fig. 4) and all three patients with negative microbiological findings and near normal granulocyte function (Fig 1 triangles) received sulphonamides and hydrocortisone in dosages as those used for patients with proven bacterial meningitis. None of the patients with bacterial infections received methimazole, colchicine, phenylbutazone, or other drugs known to interfere with granulocyte function (8 19 27) and none were diabetic (28)

Since the present technique measures the combined function of all the phagocytic cells in the blood sample, deviations from normal function may be caused by a subpopulation of defective cells similar to the situation in heterozygous carriers of C.G.D (29) (Fig 1) In bacterial infections, young myeloid cells might represent such a defective cell population and it is therefore interesting that the degree of impaired granulocyte function was significantly positively correlated to the degree of "shift to the left" (Fig 3) Little is known about differences in bactericidal capacity in normal myeloid cells at different stages of development. Myeloperoxidase is contained primarily in the azurophilic or primary granules which are abundantly present in normal promyelocytes and myelocytes, but this does not preclude malfunction of myeloperoxidase-mediated systems in immature neutrophils (2) In several patients with noninfectious granulocytosis, decreased staphylocidal function could be demonstrated (Fig 1) although not as pronounced as in bacterial infections in this group, however the proportion of immature cells was not as high as in bacterial infection (Table 1)

The possibility of a direct effect of the invading micro-organisms upon granulocyte function should likewise be considered. Granulocytes from patients with bacterial infection actively reduce nitroblue-tetrazolium *in vivo* (22) and exhibit increased oxygen consumption and hexose monophosphate shunt activity both resting and phagocytizing, *in vitro* (16) These alterations suggest *in vivo* activation of granulocyte metabolism which may be mediated by the interaction of the cells with immune complexes, formed by reaction of bacterial antigens with antibodies (10 21) It is unclear how such activation could cause functional impairment of staphylocidal activity but re-arrangement of cellular structure, competition for or utilization of one or more components of the myeloperoxidase mediated systems could presumably occur

Effective intraleucocytic killing of invading micro-organisms is as critical as effective ingestion. It therefore seems important that a selective defect in the initial killing function can now be demonstrated to result from severe bacterial infection. The question naturally arises to what extent this deviation from normal granulocyte function may hamper the efforts of the host to overcome the infection. It appears from these studies that the normal response of the host to the challenge of a serious bacterial infection may lead to defects in one of many important host resistance factors which may contribute to a fatal outcome. Further studies of this defect should provide insight into the role of circulating neutrophil granulocytes and their behaviour during bacterial infections. It should finally be emphasized that in view of the present findings the presence or absence of active bacterial infection must be taken into consideration when deviations from normal granulocyte function are disclosed in individual patients.

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DELAYED HYPERSENSITIVITY AND ESSENTIAL HYPERTENSION

Delayed Hypersensitivity Against Arterial Wall Components as a Pathogenetic Factor in Essential Hypertension

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By means of the leucocyte migration technique it is demonstrated that the migration indices from 9 patients *suffering from essential hypertension are separated significantly* from 14 patients suffering from secondary hypertension, diabetic angiopathy periarteritis nodosa, temporal arteritis, endarteritis obliterans and from 11 normal persons. *This means* that a simple secondary degenerative damage of th vascular bed is not sufficient to produce measurable cellular antibodies against the vascular wall accordingly the positive finding in patients with essential hypertension seems to be specific and possibly an important pathogenetic factor in at least some cases of essential hypertension

A hypersensitivity of the delayed type directed against arterial antigens seems to be involved in cases of essential hypertension (*Olsen* & *Loft* 1973a, b) A decisive problem is whether the hypersensitivity is only a secondary phenomenon to the hypertensive damage of the arteries and/or arterioles or whether the hypersensitivity possibly is a pathogenetic factor in essential hypertension If the first possibility is true it is to be expected that other sorts of degenerative angiopathy show the same results, using the leucocyte migration technique, as in cases of essential hypertension.

Therefore, the aim of the present work has been to compare the results obtained by the leucocyte migration technique in patients suffering from essential hypertension and other sorts of degenerative arterial disease in order to evaluate the specificity of the delayed hypersensitivity in patients suffering from essential hypertension.

METHOD AND MATERIAL

As mentioned abov the leucocyte migration technique was used (*Søborg & Bendixen* 1967 *Bendixen & Søborg* 1969)

1 *The Preparation of the Antigen(s)*

A femoral artery without macroscopical atherosclerosis from a man who had been dead for about twelve hours was used for the production of arterial antigen( ) *After* skin incision on the medial side of the thigh the femoral artery was removed under optimal aseptic conditions Fragments of the artery including tunica intima, media and adventitia were subsequently homogenized immediately by homogenizer pestles. The homogenization time was five minutes at 4 C. Finally the homogenate was extracted in Hank buffered salt solution over night at 4 C. After centrifugation the supernatant was pipetted off from sediment, and the concentration of the protein in the supernatant was measured.

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2. *The Leucocyte Migration Technique*

The migration of the leucocytes from patients suffering from essential hypertension, secondary hypertension, diabetic angiopathy temporal arteritis, periarteritis nodosa, endarteritis obliterans, as well as the migration of leucocytes from normal persons was studied in culture chambers containing an arterial protein concentration of about 50 micrograms per ml. The results were given as the migration index, which is defined as the ratio between the migration area of antigen-containing and antigen-free cultures.

The precision of the method expressed as coefficient of variation was 5 per cent.

3 *The Patient Material*

The experimental groups were as follows

A Nine patients suffering from essential hypertension, the group comprising five women and four men. The age varied from 26 to 75 years and the blood pressure from 160/110 mm Hg to 230/140 mm Hg. Only two patients were treated with antihypertensive drugs at the time of the study On the basis of X-ray of the thorax, the electrocardiogram, subjective and objective symptoms from the heart, the cerebrum, and legs only one of the nine hypertensive patients showed signs of arteriosclerotic lesions of the arteries.

B. Six patients suffering from secondary hypertension, four on account of renovascular anomalies, one on account of unilateral renal disease and one on account of thyrotoxicosis. The group comprised three women and three men. The blood pressure varied from 170/120 mm Hg to 240/140 mm Hg and the age from 28 to 59 years. None of the patients was treated with antihypertensive drugs at the time of the study

C Five patients suffering from diabetes mellitus for many years and treated with insulin. All the patients showed marked signs of diabetic angiopathy localised to the large arteries, the kidneys and the retinal vessels. The age of the patients varied from 29 to 59 years and the group of patients comprised three women and two men. Their blood pressure was normal.

D Three patients who suffered from biopsy verified periarteritis nodosa, temporal arteritis and endarteritis obliterans localised to the legs and the abdomen. None of the patients was treated with drugs at the time of the study The age varied from 43 to 72 years. The blood pressure was normal.

4. *The Control Persons*

Eleven healthy persons without arterial hypertension or other demonstrable degenerative vascular disease. The age varied from 22 to 59 years and the group comprised five women and six men.

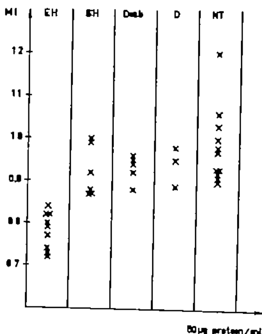


Fig. 1 Abscissa. The arterial protein concentration in the culture chambers. Ordinate Migration index (MI) EH Essential hypertension. SH: Secondary hypertension. Diab: Diabetic angiopathy D Temporal arteritis, periarteritis nodosa, endarteritis obliterans. NT: Normotensive controls without signs of degenerative angiopathy

The figure shows that the migration indices from the patients suffering from essential hypertension are clearly separated from those of patients in the other four experimental groups.

RESULTS

The results obtained by the leucocyte migration technique from the different experimental groups are seen in Fig. 1 It shows that the migration indices from patients suffering from essential hypertension are clearly separated from the indices of patients suffering from secondary hypertension, diabetic angiopathy temporal arteritis, periarteritis nodosa, endarteritis obliterans and from the indices of normotensive controls. If patients with essential hypertension are compared with those in one of the other experimental groups a significant differences between the migration indices is found in all cases, $P<0.005$ (Wilcoxon Mann Whitney rank sum test)

DISCUSSION

The results show that the migration indices from patients suffering from essential hypertension are separated significantly from the migration indices from patients suffering from the other sorts of degenerative angiopathy. This means that a simple (secondary) degenerative angiopathy seems not to be sufficient to produce measurable cellular antibodies against the arterial wall by the method used. Therefore it is likely that a delayed type of hypersensitivity directed against unknown antigenic substances in the arterial and/or arteriolar wall is an important, maybe a causal, pathogenetic factor in at least some cases of essential hypertension.

The fact that a hypersensitivity of the delayed type directed against the arterial wall can develop following an experimental hypertension has been described previously (*Olsen* 1970 1971 *Svendsen* 1973) and these experiments showed among other things that substances in the arterial wall could have an auto-antigenic function under these experimental circumstances.

Besides essential hypertonics only arteriosclerotic patients have shown a delayed hypersensitivity against the arterial wall (*Loft* & *Olsen* 1973) when the value of the leucocyte migration technique was studied in cases of different sorts of angiopathy.

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BRIEF REPORTS

ACCIDENTAL NOSEMATOSIS IN MICE WITH IMPAIRED IMMUNOLOGICAL COMPETENCE

Gunnel Huldt and T. Waller

Animal experiments that influence the immunological competence imply a risk for secondary infection with so called opportunistic agents which are normally controlled by intact defense mechanisms. The manifestations of such often unexpected and not always recognized infections may sometimes lead to confusing results. This is true particularly when the complicating agent is one that attacks and causes reactions from the immune apparatus.

It has been demonstrated that experimental infection with *Toxoplasma gondii* (tx) is accompanied by depression of the immunological reactivity to a number of unrelated antigens including various infectious agents (3, 6, 7). Also Moloney virus (MLV) has been shown to exert an immunodepressive effect (4).

In the course of an experimental study of double infection with tx and MLV a group of mice developed acute nosematosis with symptoms that could initially not be clearly distinguished from either one of the two experimental infections.

A litter of C57Bl mice, born 14 June 1972 was inoculated on the day of birth with tx and MLV. Tx was given as a suspension of brains from Swiss albino mice (kept in a separate animal unit) and infected 3 months earlier; the inoculate containing 3–5 cysts per infective dose. The virus inoculate was a cell free homogenate of MLV infected lymphoid cells obtained from the Dpt of Tumour Biology, Karolinska Institutet Medical School, Stockholm.

Three mice died before the age of 4 weeks. A mouse taken at the age of 5 weeks did not show anything unusual at autopsy and the histopathological picture of lymphoid organs (spleen, lymph nodes, thymus) did not reveal any changes that could not be referred to the tx + MLV infection.

A mouse taken at the age of 5 weeks, however had a small peritoneal exudate and an unusually large spleen (236 mg). Within the next days the abdomen of the two remaining mice was rapidly extruding. One of these mice was taken at the age of 37 days and one at 6 weeks of age. The findings in both were similar. The animals had large peritoneal exudates, 3 and 5 ml respectively; one of them also a pleural exudate. Both liver and spleen were enlarged. The weights of the spleens were 298 mg and 316 mg, respectively, which is significantly higher than the mean spleen weight in tx + MLV infected 6 weeks old C57Bl mice (143 ± 21 mg). The livers contained yellowish grey spots of an appearance not seen in either one of the two experimental infections.

The exudates from the three mice contained large numbers of macrophages. Cytocentrifuge preparations of the cells were made according to *Doré & Balfour* (1) and were stained with Giemsa and also with a FICT-conjugated rabbit-antitoxoplasma serum (Wellcome lot K5014). Aliquots of 0.5 ml were injected ip in fresh clean 8 weeks old C57Bl mice for isolation experiments. The mice developed within 3 weeks large peritoneal exudate. They all had marked splenomegaly and remarkably small thymus glands. Toxoplasma was not demonstrated by any of the techniques used (including dye test 4 weeks after inoculation). The exudate cells also were incubated with anti-MLV serum and complement. No cytotoxic effect of the serum was demonstrated.

After renewed careful examination of the Giemsa stained preparations, however, it was found that a small number of the peritoneal macrophages contained organisms which were about 1.5–2.5 μ and elongated or ovoid. In preparations stained according to *Wright & Craighead* (8) they appeared bluish-red. They were found to be dark blue when stained by the Goodpasture method (2) and they were also found to be Gram-positive. Conclusively the diagnosis of *Nosema cuniculi* (*Encephalitozoon cuniculi*) infection was made.

Nosema organisms were isolated in secondary calf kidney cells and in established cell lines of rabbit kidney cells as well as of feline lung cells origin. So far the nosema organisms have been kept up to 40 subpassages in the cell cultures. Mice

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Fig 1 Intracellular *Nosema cuniculi* spores in rabbit kidney cell culture isolated from infected mouse (Wright & Craighead, × 800)

which were intraperitoneally inoculated with material from the nosema infected cell cultures developed ascites within 1–3 weeks.

In the tx + MLV infected C57Bl litter the first clinical manifestation of the complicating nosematosis was development of peritoneal exudate containing microsporidiae. This indicates that the mice might have been infected ip and if so that the tx + MLV inoculate probably was contaminated with nosema. It is most likely that the toxoplasma containing brain suspension was the contaminated component. The brain is one of the main locations of the parasite in chronical nosematosis. Furthermore it was shown that a rabbit caged in the same room as the donor mouse had nosematosis with parasites in the kidneys. It is not unlikely that this animal by excreting the parasite with the urine also did infect other animals in the room.

Suspension of brains both from cagemates of the donor mouse and from other toxoplasma infected mice in the same room were inoculated ip into several groups of Swiss albino mice. Considerable peritoneal exudate or other signs of disease were not seen in any case and no deaths occurred. The low virulence toxoplasma strain used for inoculations gives very little exudate. Even if it is taken into consideration that Swiss albino mice may be less susceptible to nosema infection than C57Bl, it cannot be excluded that the poor immunological capacity of the C57Bl litter might have influenced the outcome of the infection in these mice. When inoculated they were immunologically immature and later immunosuppressed by the double infection.

The here reported accidental appearance of nosematosis is certainly not an unique occurrence. A number of reports of similar observations have recently been summarized by *Shadduck & Pakes* (5). *Nosema cuniculi* is common among laboratory animals, both rabbits and mice. As the agent is found in the urine it can easily be transmitted from one laboratory animal to another unless very strict preventive measures are taken. The maintenance of various infectious agents as certain viruses and low virulence toxoplasma by passage of host material to new mice certainly contributes to the spread within laboratory animal units.

Available data indicate that chronical nosematosis does not give many clinical symptoms (5). Whether impaired immunological reactivity can activate the infection is not clear but the present observation may speak in favour of this supposition. It is desirable that systematic studies are initiated to clarify this question.

The extensive use of mice in microbiological experiments, where a complicating nosematosis may influence the results, justifies further studies on the effect of this agent on laboratory animals.

This work was supported by grants 72.8 from the *Swedish Cancer Society* and 3646 from the *Swedish Medical Research Council.*

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ISOLATION OF HAEMOLYTIC *ACTINOBACILLI* FROM HORSES

J. L. Larsen

Actinobacillus equuli has been encountered as a very common micro-organism causing neonatal infections in foals. This micro-organism could contribute to 25 per cent of all infections in this period of life, and under certain circumstances to a mortality rate of about 100 per cent (*Blood & Henderson* 1968). While the pathogenicity for foals is very high (*Baker* 1972) *A. equuli* in other animals, e.g. rabbits (*Arseculeratne* 1962) and pigs (*Ashford & Shirlaw* 1962, *Jones & Simmons* 1971 *Cutlip et al.* 1972 *Windsor* 1973 *Mair et al.* 1974) could be regarded as an opportunistic pathogen implicated in different infections. Most infections with *A. equuli* in pigs have been recorded in the age group 1 to 6 weeks, but also sows could be attacked. The purpose of this communication is to record the isolation of haemolytic *Actinobacilli* that infrequently fermented mannitol. The bacterial strains were isolated during routine microbiological diagnostics. Isolation was made mainly from autopsy material. Six strains were isolated from foals with septicaemia, one strain from a four-week-old foal with arthritis and two strains from horses with a chronic alveolar emphysema. The bacterial cells were gram-negative non-motile, pleomorphic rods, varying from coccobacilli, sometimes in short chains, to long slender rods.

Colonies on calf-blood-agar after 24 hours of incubation at 37° C were 1–2 mm, raised, circular and entire. They adhered to the agar surface and were sticky when picked with a needle. The colonies were surrounded by a very sharp, approximately 2 mm broad haemolytic zone. Serum enhanced the growth and litmus milk was slowly acidified and slimy acid. The growth in broth media was viscous. The strains were sensitive to sulphonamides, chloramphenicol neomycin, streptomycin, polymyxin, erythromycin, tetracycline and moderately sensitive to penicillin. The haemolytic properties could not be transferred to an *Escherichia coli* K 12 F⁻ (*Smith & Halls* 1967) and the haemolysin was not soluble when tested according to *Smith* (1963). In horse kidney cells, the haemolytic *Actinobacilli* demonstrated a considerable cytopathic effect, in contrast to a non-haemolytic *A. equuli*. Preliminary serological tests demonstrated a certain degree of similarities between haemolytic and non-haemolytic strains. The strains were pathogenic to mice. Except for the haemolysis and the mannitol fermentation, all other criteria correspond to the descriptions of *A. tinobacillus equuli*. (*Bergey's Manual* 1957 *Cowan & Steel* 1966). However *Van Dorssen & Jaartsveld* (1962) *Wetmore et al.* (1963) *Zimmermann* (1964 & 1965) and *Bouley* (1966) isolated strains with similar biochemical properties from pigs.

The name *Actinobacillus suis* has been proposed, independently by *Van Dorssen & Jaartsveld* (1962) and *Zimmermann* (1964). But as the main differences are manifest only in two criteria, and the pig is not the only host, the conditions are the same as those between haemolytic and non-haemolytic *E. coli*. However *Frederiksen* (1971 and 1973) found that the haemolytic strains of *Actinobacilli* demonstrated further discrepancies. The uniformity of the carbohydrate fermentation could not be demonstrated in strains isolated from horses. The conditions for creation of the new species name *A. suis* may be discussed, as the pig is not the only host of these micro-organisms.

M ay (1968) also described haemolytic *Actinobacilli* in horses and, on the basis of examinations of the guanine-cytosine per cent and additional serological and biochemical investigations, he proposed to retain *A. equuli* as species name.

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Available data indicate that chronical nosematosis does not give many clinical symptoms (3). Whether impaired immunological reactivity can activate the infection is not clear but the present observation may speak in favour of this supposition. It is desirable that systematic studies are initiated to clarify this question.

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of each (25 ml) was then centrifugate separately until it became limpid. The supernatants were mixed each with 0.5 ml of freshly drawn venous blood (stabilized with 1 per cent sodium citrate) from a sheep suffering from heartwater (verified later at autopsy) and then poured back to the flasks of origin. The presence of infective *C.r* organisms in the blood was verified by injection of 5 ml of the latter into a susceptible sheep which sixteen days later died from typical heartwater. After inoculation, the cultures were then incubated at 37 C for 8 h, upon which the medium blood *mixture was discarded. The cultures were then* rinsed for remaining blood by gentle washing three times with 20 ml of medium. To each culture was *finally added 25 ml of conditioned* medium which had been preconditioned with an extract of tick *eggs (1) and incubated at 26 C.*

In the following incubation period, cover slips were taken daily from the flasks, stained according to Giemsa, and examined for the presence of microorganisms and cellular changes. From day 4 following inoculation, granules were observed in cells from some of the cultures. Approximately 5 per cent of the cells contained on an average about 10 distinct granules in their cytoplasm. At days 6 7 and 8, the percentage of infected cells in the said cultures was: 15 per cent, 22 per cent and 65 per cent, respectively The number of granules in each infected cell was steadily increasing. At day 8, more than 50 granules were counted in many cells. *The granules became dark purple after staining* with Giemsa, their diameters varying from 0 1 µ to 1 µ. They all appeared to have an intracellular position and seemed to correspond fully with the specific *C.r* granules described by *Cowdry* (2) No cellular changes were observed until day 9 of incubation when some of the cells started to degenerate. Appearance of granules, followed by a numerical increase of these and cellular degeneration, was only observed in a group of cultures which prior to incubation had shown a high mitotic activity (mitotic index of about 25 as measured on cell layers stained with acridine orange)

Two positive, nine-day-old cultures were suspended and each injected intravenously into a susceptible sheep. One of the sheep died 12 days after injection, the other 15 days after injection. The clinical symptoms observed in both sheep were typical of heartwater At autopsy also the gross-lesions were typical of the said disease. By histological examination of brain squashes, stained according to Giemsa, the typical *C.r* organisms were recovered in both cases. One sheep, which was injected with a four-day-old positive culture (about 1 per cent of the cells in the culture was "infected" with an average of about 5 granules per cell) did not react. Injection of cell cultures exposed to *C.r* negative blood and unexposed cultures did not give rise to disease in the control sheep.

All the sheep were regularly sprayed with acaricides and kept in tick proof pens in order to exclude natural transmission of the disease (only by vector ticks)

*The inoculum used for infection of the cell cul*tures corresponds to half the amount of the lowest dose described to be infective for sheep (5) *The high dilution of infective particles, ob*tained by washing the cell cultures for the infective sheep blood *after the 8 hours incubation,* together with the very high fragility of the *C* organism (e.g survival 4 h at room temp. and 24 h at 4 C) excludes a survival of a number of infective particles sufficient to enable a single culture to infect a sheep after nine days.

Thus, it seems that *C.r* positive sheep blood can infect the above mentioned tick cells in vitro and that *C.r* organisms are propagated in these cultures to such a degree that an infective dose of *C.r* sufficient to provoke heartwater in sheep, may be obtained in each culture within, at least, nine days.

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STUDIES ON *STACHYBOTRYS ALTERNANS*

IV Effect of low doses of stachybotrys toxins on pregnancy of mice

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Low doses of stachybotrys toxin were administered per os to groups of mice in early pregnancy. The toxic material was given either in the form of infected grain, liquid growth medium or partly purified toxin preparation. The administration took place mostly either as a single dose on the 3rd or 5th day of pregnancy or during a five-day period in the feed. The toxin amounts administered varied from 3 to 4000 tissue culture units (TCU) measured by the mouse fibroblast tissue culture test. The proportion of pregnant mice in all toxin treated groups was 70.7 per cent and that in the control group was 90.5 per cent. The difference is statistically significant. Statistically significant differences were also demonstrated in the frequency of dead, resorbed and stunted foetuses and in the average litter size of live normal foetuses between the control group and the groups administered 100–4000 TCU of the stachybotrys toxin. The results thus provide experimental evidence that stachybotrys toxin can affect detrimentally foetuses in doses low enough not to cause any definite clinical signs of illness in pregnant females. The histopathological study revealed uteroplacental haemorrhages in toxin treated animals. This finding may be indicative of the mechanism of action by toxin.

The effects of drugs and toxic substances on the foetal development have attracted growing attention in the last decades.

The aetiology of numerous abortions, stillbirths and cases of infertility still awaits its clarification both in human and veterinary medicine. Several mycotoxins, such as ergotoxins (*Mantle & Finn* 1971) ochratoxin A (*Still et al.* 1971) and aflatoxins (*DiPaolo et al.* 1967) have been experimentally demonstrated to be able to interfere with the normal course of pregnancy. Anamnestic data connected with the isolation of *Stachybotrys alternans* Bon. [syn. *S. atra* Corda, *S. chartarum* (Ehrenberg ex Link) Hughes] strains in Finland (*Korpinen & Ylimäki* 1972, *Korpinen & Uoti* 1974) suggested to the present author that stachybotrys toxins might also exert a similar effect. *Forgacs & Carll* (1962) and *Vacher et al.* (1970) have reported field data indicative of involvement of stachybotrys toxins in one case each of mare and sow abortion. The present paper describes the results of experiments where low doses of stachybotrys toxins were administered to mice mainly in the early phase of pregnancy.

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MATERIAL AND METHODS

A. Test Mice

The white mice used in the experiment were of CFW strain, the females weighing 20–25 g and the males 25–30 g. The females had their second or third gravidity. The males were sexually experienced. The males were placed together with the females (2:10) at night and removed 11–12 hours later. The females with the vaginal plug were

regarded as gravid. The gravidity was estimated to be 0–12 hours old by this time.

The animals were fed a commercial mouse diet (*Astra*, Sweden) as their basic diet and they received water ad libitum.

The animals for control groups were randomly selected from the mice having a vaginal plug. All mice were killed with ether on the tenth day of the pregnancy except for Group 14, where this was done on the 15th day. The living, dead, resorbed and stunted foetuses were counted and the appearance of the uterus was recorded.

B. Preparation of Toxic and Control Materials

Five *S. alternans* strains were employed. Strains 71, 76 and 87 were toxicogenic, strains 83 and 86 non-toxicogenic. The toxic substances were either administered without any purification procedure in the form of infected grains (sample 82) or as a toxin-containing liquid synthetic growth medium (sample 76), or as a partly purified preparation (sample 71).

Strain 71 was grown on a grain mixture (barley, wheat, oats 1:1:1) in the routine way (*Korpinen* 1973). Extraction and purification of the toxin were accomplished with ether, petroleum ether and silica gel column runs with acetone-benzene processing as described earlier (*Korpinen et al.* 1974). The resulting partly purified stachybotrys toxin solved in distilled water was used at a dry substance concentration of about 1 mg/ml.

Strains 76 and 83 were grown in a liquid modified Czapek medium for two months. This medium, with agar included, has been described earlier (*Korpinen & Uoti* 1974). The liquid media after the growth of strains 76 and 83 as well as the extract of strain 71 toxin were sterilized through a Millipore filter with pore size 0.2 μm before being subjected to biological testing. The toxicity of the preparations was quantitatively measured by using mouse primary fibroblast cultures in the manner described earlier (*Korpinen et al.* 1974). Series of tenfold dilutions of the preparations were prepared and tested. The highest dilution producing a distinct positive reaction in the tissue culture (toxicity grade 3) was called one tissue culture unit (TCU).

The tissue culture toxicity tests of the preparations concerned were repeated 2–3 times. The titres remained unchanged.

Strains 82 and 86 were grown for two months at room temperature on the grain mixture already stated. Before feeding, the grain materials were finely ground and 5 per cent mixtures with balanced mouse diets were prepared. One of the control feeds used was an uninoculated grain mixture autoclaved to sterility and mixed with the diet as above.

C. Administration of the Test and Control Materials

Each test group of pregnant mice comprised 8–10 animals. The toxin dosage for this study was found by trying out several dosage levels. The toxin quantities received by the groups fed toxic grains or toxic liquid medium of Group 5 are only approximate because the mice ingested this diet somewhat reluctantly. According to the toxin dose administered and the toxin preparation used, the test groups belong to one of three main classes of treatment. It was found in preliminary tests that a single dose of 10,000 TCU killed the animals.

Table 1 contains data on the manner and time of administration and on the amount of TCU of toxin administered. When the sample was administered as a single dose, the mice were slightly anaesthetized with ether and the dose was given through a stomach tube in 1 ml of water.

Five groups of mice served as the controls. The main data concerning them appear in Table 1. Two of the groups (Groups 4 and 7) received materials prepared from grain or liquid medium infected with non-toxicogenic *Stachybotrys* strains, while one group (Group 8) was given non-infected grain mixture. The two remaining control groups had nothing but the basic diet, and one of them (Group 15) underwent the same anaesthetic procedure on the 3rd or 5th day as the groups administered toxic materials. No special treatment was given in the other group (Group 9).

D. Histopathological Studies

All the animals of control Group 9 and of the toxin Group 12 served as material for histopathological studies. One or several tissue specimens taken from one or both uterine horns were fixed in 10 per cent formalin for 1 month and embedded in paraffin in routine manner. Sections made at 5μ were stained with haematoxylin and eosin (HE). Specimens of tissue from the liver and kidney of one mouse of the control group and one of Group 12 were also histologically examined.

RESULTS

Table 1 shows the data concerning number of non-pregnant animals and the number of affected and normal foetuses in the groups of mice treated with varying doses of stachybotrys toxin in different manner. Equivalent data are also stated for the different control groups. Table 2 gives the data concerning differences in relative frequency of the above phenomena between the combined control group and the major test groups

TABLE 1 *Effect of Different Stachybotry T xin Doses on Pr gnancy of Mic*

Group No	Number of females	Dose TCU	Adm on day	Strain No	Non-pregn. females	Numbers in the group: Stunted foetuses	Numbers in the group: Dead Resorbed foetuses	Normal foetuses	Mean number of normal foetuses per female
Contr									
4	8	0	5	83	1	1	5	62	6.9
7	9	0	1–5	86	1	1	7	61	7 6
8	8	0	–	*	0	8	1	80	9.6
9	9	0	–	†	1	3	1	73	8.1
15	8	0	–	‡	1	1	2	67	8.4
I									
1	8	2000	3	71	5	0	1	22	2.8
10	8	2000	5	71	1	15	8	33	4 1
12	8	4000	3	71	2	2	3	23	2.9
II									
2	10	100	3	76	5	0	6	54	5.4
3	8	100	5	76	1	12	5	49	6.1
III									
6	8	500/day	1–5	82	0	15	6	31	3.9
13	8	500/day	5–9	82	4	21	5	17	2.1
14	9	500/day	10–14	82	4	0	7	38	4.2
5	8	5/day	1–3	76	0	6	4	71	8.9

* = 5 per cent control feed.
† = normal diet.
‡ = narcosis control.

the frequency of affected foetuses between the groups of treated and control animals is considered evidence of the effect of stachybotrys toxin. The histopathological finding of uteroplacental haemorrhages in the mice of Group 12 lends additional support to this interpretation. This finding also provides the only direct clue as regards the mechanism of stachybotrys toxin effect leading to deaths and stunting of foetuses. An indirect way of influence through disturbance of the foetal circulation is thus suggested. The mechanism of a direct effect exerted by the toxin on the foetuses seems to be opposed by frequent presence of affected as well as unaffected foetuses in the same uteri.

The ability to cause haemorrhages is a well-known characteristic of stachybotrys toxin. In the present experiments the haemorrhages were confined to the uterus, placenta and foetal membranes. It is tempting to speculate that the developing blood vessels of the fast-growing uterus are a target more sensitive than other tissues to the attack by stachybotrys toxin.

Several mycotoxins are known to exert a harmful effect on pregnancy. The question arises to what extent the mechanisms of influence could be similar to that of stachybotrys toxin. A variation in mechanism according to type of mycotoxin is apparent. F 2, zearalenon, causes infertility in domestic animals through its oestrogenic effect (*Mirocha et al.* 1971). Certain ergot alkaloids are known to prevent implantation in mice (*Mantle* 1969). Ochratoxin A has been reported by *Still et al.* (1971) to cause foetal death and resorption, and a uterohaemorrhagic effect in the rat. The few studies on aflatoxin contain contradictory results with reference to the present discussion. *Le Breton et al.* (1964) reported uteroplacental haemorrhages and foetal death in rat and mice on intraperitoneal administration of aflatoxin. *Butler & Wigglesworth* (1966) only recorded foetal retardation in rats given aflatoxin B_1 orally. *DiPaolo et al.* (1967) again, reported the occurrence of changes similar to those found earlier by *Le Breton et al.* (1964).

Occurrence of uteroplacental haemorrhages has thus been reported in connection with effects both of ochratoxin A and aflatoxin. Whether these facts imply a similarity in the mechanism of their foetal effect with that of stachybotrys toxin cannot be said with the present understanding.

The frequently occurring fertility problems of the type in which the animals are clinically healthy remain unclarified in the field at present. According to *Roberts* (1971) the causative agent of abortions is only diagnosed in 20–23 per cent of cattle abortions in areas where brucellosis is rare, and the corresponding number of swine abortions is 25–50 per cent. Mycotoxins in general are considered, with good reason, to bear some of the responsibility. The results of the present experimental study lends additional support to the hypothesis that a proportion of the fertility problems of the domestic animals in the field could be explained in terms of stachybotrys intoxication.

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STUDIES ON *STACHYBOTRYS ALTERNANS*

V Comparison of rabbit skin mouse fibroblast culture and brine shrimp tests as detectors of stachybotrys toxins

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Three types of biological methods were compared as detectors of stachybotrys toxin. The seven preparations tested represented "crude" or partially purified toxins produced by different strains of *Stachybotrys alternans* and toxic fractions obtained by partial purification of the product of one strain. The order of sensitivity of the methods was almost invariably the mouse fibroblast test most sensitive, the rabbit skin test next, and the brine shrimp (*Artemia salia*) test last. A conspicuous variation by preparation in relative sensitivity of the mouse fibroblast test and the rabbit skin test was revealed, however The variation ranged from a sensitivity of the rabbit skin test approximately 2.5 times higher than that in the mouse fibroblast tests to the other limit when the mouse fibroblast test was 80 times more sensitive than the rabbit skin test. The variation is interpreted to provide the first experimental proof of heterogeneity in biological effect based on chemical differences among the compounds constituting "stachybotrys toxin The toxic fractions obtained by purification of tw crude preparations and identified as toxic by the mouse fibroblast test showed different patterns of distribution of toxicity The results demonstrate that qualitative differences may exist in the chemical structure of the population of toxic compounds of stachybotrys toxin produced by different strains of *S alternans*.

A variety of biological methods are being used for qualitative and quantitative studies of mycotoxins. The results are naturally affected by the sensitivity of the method and, particularly in analytical enquiries into the nature of the mycotoxin complex, also by the extent of coverage of mutually differing toxic compounds. The diversity of methods applied in the studies of a particular mycotoxin and the lack of comparative methodological studies often make it difficult to compare the results obtained in different laboratories. Such is the present situation also in stachybotrys research.

The methods principally relied on by the research workers studying this toxin have been the rabbit skin test (*Palyusik & Juhasz* 1967 *Sarkisov et al.* 1971 *Pathre et al* 1973) the tissue culture test (*Nummi & Korpinen 1973*) and the brine shrimp test (*Eppley* 1973) So far no comparative studies of these methods have been published.

The main purpose of the present study was to gain an understanding of the relative sensitivity of the said types of methods in stachybotrys toxin research. Furthermore, the possibility of qualitative differences between the methods in the detection of specific components of different stachybotrys toxin preparations was investigated.

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MATERIAL AND METHODS

A Toxin Preparations

Both "crude" and various partially purified stachybotrys toxins were used. All stachybotrys toxin preparations were produced by culturing, as the first step, toxin-producing *S alternans* Bon. [Syn *S atra* Corda, *S chartarum* (Ehrenb. ex Link) Hughes] strains in a grain mixture (wheat oats barley 1 1:1) The two crude toxins No. 3 6 and 82 (numbered according to the producing strain) were petroleum ether precipitates of ether extracts of infected grains, obtained by extraction in a Soxhlet apparatus (for technique refer to *Korpinen* 1973)

Preparations 71 and 72 were derived from crude toxins of the respective strains by silica gel column runs with acetone benzene 1:1 (v/v) (for technique refer to *Korpinen et al.* 1974) and by collection of the toxic fractions. For detection of toxicity the mouse fibroblast culture test was applied. The toxic fractions comprised in the preparation 71 all emerged from the column as one single peak, i.e., they formed a continuous series of tubes.

In contrast, the toxic material collected in preparation 72 was eluted as two peaks separated by non-toxic fractions. The material 72 a, comprising the first of the two, the main toxic peak, was further fractionated and purified by silica gel column runs with chloroform-petroleum ether methanol as eluent. The resulting two separate series toxic fractions are referred to preparations 8I and 8II in this paper according to a terminology adopted in an earlier work (A detailed description of the pattern of distribution of toxic fractions comparable with that of preparation 72 is given in Fig 1 in *Korpinen et al.* 1974) The second, minor toxicity peak, resulting from the primary purification of preparation 72, forms the preparation 8 III of the present study

For the purposes of the rabbit skin test and brine shrimp test acetone (pro analyse grade) solutions, and for the mouse fibroblast test solutions in normal saline, were prepared. The primary solutions were further diluted in steps of 1:10 in acetone or saline respectively to allow a crude quantitation to be made for each type of test. For more accurate titration series of two fold dilutions were prepared.

Verrucarin A and roridin A were included in the tests as representatives of trichothecene mycotoxins. They were obtained in powder form by the courtesy of Prof. Dr *Ch. Tamm* Basel (Institut für Organische Chemie der Universität) The dilution series were prepared as for the stachybotrys toxin preparations.

B. Rabbit Skin Test

The test was carried out as described by *Forgacs et al.* (1958) and *Sarkisov et al.* (1971) except that the toxic preparations were solved and diluted in acetone instead of saline. The albino rabbits used, both males and females, weighed about 2.5 kg Four to five serial dilutions of a toxic preparation were applied, 0.05 ml of each, to a spot on a shaved flank of the rabbit.

The application was carried out dropwise by a micropipette allowing each drop to dry before the next one was placed. A control spot of acetone was included in each experiment.

The reactions were recorded daily on five days following application. The highest dilution which caused a detectable inflammatory reaction of the skin during any day of the 5-day period was recorded as the toxin titre of the preparation. Most often there appeared a red spot on the skin within one day after the application of a dilution which was to prove positive The changes produced ranged from a light red scaly area of the skin to obvious necrosis (Fig. 1)

C Mouse Fibroblast Culture Test

The test was performed as described earlier (*Korpinen & Uoti* 1974) The inoculum was 0.1 ml. Occasionally when stained preparations were desired, cover slips were placed on the bottom of petri dishes and further processed as also described earlier

The highest toxin dilution which showed the toxicity grade of 3 (*Korpinen et al.* 1974) was designated as the titre of the preparation.

D Brine Shrimp Test

Brine shrimp (*Artemia salina*) "eggs" (dried encysted gastrulae) originating from Tunder USA or from ordinary aquarium shops in Helsinki were hatched in artificial sea water (5–7 per cent salt) at room temperature (22–24 C) Two to three teaspoonfuls of eggs were inoculated into 1 liter of water Air was usually conducted into the water in small bubbles through a tube although it was found that the hatching also succeeds without aeration. Three days after the emergence of first nauplius larvae the hatched larvae were used as test animals (Fig. 2) without prior separation of unhatched eggs from the batch. In order to attain the desired concentration of the larvae, they are filtrated through ordinary filter paper and resuspended.

In the test 0.1 ml of toxin dilution was placed into each test tube, the acetone was evaporated and an estimated 40–100 *Artemia salina* larvae in 2.5 ml saltwater were transferred into the tubes. The tubes were kept at room temperature (22–24 C). Control tubes with 0.1 ml of acetone and several tubes without acetone were always included in the experiments.

The affected *Artemia* larvae were immobilized and sank to the bottom. This phenomenon was relied on as the criterion of positivity in conformity

brine shrimp test was invariably least sensitive. It is of great interest however that the relative sensitivity of the mouse fibroblast and rabbit skin tests clearly varied with the preparation tested. This is considered to constitute a qualitatively new type of support for the theory of heterogeneity of stachybotrys toxin to the evidence of chemical heterogeneity (*Korpinen et al.* 1974) experimental data have now been added indicating heterogeneity in biological effects.

The results as regards the general succession in relative sensitivity revealed bring up the question of the representativeness of the present data. Are the sensitivities observed typical of the types of method? Both the mouse fibroblast test and the rabbit skin test, in the forms applied are or closely resemble *standard techniques in laboratories working* with stachybotrys toxins. The degree of representativeness of the brine shrimp test remains more questionable. The technique has been used in stachybotrys toxin studies by *Eppley* & *Bailey* (1973) and in other mycotoxin studies by *Brown* (1969) and *Brown et al.* (1968) The present technique differed in some details from their method. Comparison in regard of sensitivity can be indirectly based on the results of measurements of trichotechene type toxins (e.g roridin and verrucarin) carried out both in the present study and by *Eppley* & *Bailey* The present modification of the brine shrimp test thus appeared to be lower in sensitivity about 1/10 of that achieved by *Eppley* & *Bailey* The measurements of the said control mycotoxin *further indicate that the mouse fibro*blast and rabbit skin tests, as used in the present study were more sensitive than the brine shrimp test as applied by *Eppley* & *Bailey*

The variation of relative sensitivity between the mouse fibroblast test and rabbit skin test by the preparation of the stachybotrys toxin studied appears to be major finding of the present study (Because of the lower accuracy of the brine shrimp test the respective *results* possess less value *in this* connection)

The results concerning the relative toxicities of the fractions S I S II and S III provided a more direct type of evidence for a correlation of chemical structure and biological effect. The fraction S II represents a compound or compounds of high relative toxicity to fibroblasts. The fraction S I again is quite toxic in the rabbit skin test. The nature of the fraction S III remains somewhat open but it seems to have a higher relative toxicity to fibroblasts than to the skin.

As mentioned earlier the toxic material of preparation 71 emerged as one single peak of fractions from the chromatographic column whereas that of preparation 72 came out as two peaks. This fact is evidence to the effect that different *Stachybotrys* strains produce different toxins.

These findings lead to the following conclusions a) "Stachybotrys toxin contains compounds which differ from each other in their biological effects b) The mouse fibroblast test and the rabbit skin test show variations in relative sensitivity to different toxic compounds of the stachybotrys toxin complex c) Stachybotrys toxins, as they appear in nature may vary in their composition.

The mechanisms of toxic effects by stachybotrys toxin are *far from clarified.* It may even by questioned whether direct effect of toxins on the cells is the only way of influence (*Olegović et al* 1971) The observation of phenomena such as frequent conjunctival affection of the rabbits subjected to the skin test (unpublished data) suggests the *possibility of immunopathological* mechanisms.

Eppley & *Bailey* (1973) have presented evidence that stachybotrys toxins would chemically belong to the *trichotechene* toxins Verrucarin A and roridin A were included in the present studies as representatives of the said group. In the measurement of both these toxins the mouse fibroblast test *proved* 10 times more sensitive than the rabbit skin test. The high variability of relative sensitivities when different stachybotrys toxin preparations were investigated is considered to

support the idea that compounds of other types than verrucarin and roridin are also likely to be present as toxic factors.

The author wishes to express her thanks to Professor *Ch. Tamm* Basel, for kindly supplying verrucarin A and roridin A. Mr *Markku Kurkinen*, M. Sc. prepared the purified toxin fractions. To Mrs. *Eeva Ako* and Miss *Sirikka Mäkelä* thanks are due for their good, precise technical assistance.

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FREQUENCIES OF STREPTOCOCCI OF GROUPS A, B, C, D AND G IN URETHRA AND CERVIX SWAB SPECIMENS FROM PATIENTS WITH SUSPECTED GONOCOCCAL INFECTION

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Urethral (male or female) and cervical swab specimens from 526 patients to be investigated for gonococcal infection were examined for streptococci of groups A, B, C, D and G. The overall frequencies of streptococci were group A 0.6 per cent B 19.0 per cent C: 0.8 per cent D 15.0 per cent and G 0.6 per cent. 22.3 per cent of the females and 11.7 per cent of the males harboured group B streptococci. 31 7 per cent of 41 patients with gonococcal infection harboured group B streptococci. This frequency is higher ($p < 0.05$) than those found among the other patients. These findings lend support to the view that group B streptococci in women are spread by sexual intercourse. The frequencies of streptococci group B and D in the males investigated, 11 7 per cent and 9.3 per cent, respectively are higher than those found by others. Finally of 81 females with group B streptococci, 45 7 per cent harboured group B streptococci in both urethra and cervix, 48 1 per cent only in urethra and 6.2 per cent only in cervix. Screening of women for group B streptococci should therefore always include urethral swab specimens.

The incidence of group B streptococcal meningitis in newborns has increased during the last decade (2 3 8 19) It is widely believed that such infants are infected during their descent through the maternal passages (2 3 8 19) Culture of urethral and vaginal smears obtained at term from 118 Swedish women gave growth of B streptococci in 14 per cent (5)

Bergqvist et al. (4) considered it "reasonable to perform bacteriological examination of vaginal specimens in pregnant women at term" They also felt that obstetric complications in vaginal carriers of group B streptococci indicate antibiotic therapy Some authors even recommend such treatment of all such mothers (15)

This paper concerns the epidemiology of group B streptococci among adults, as judged from the frequencies of beta-hemolytic streptococci of groups A, B, C, D and G in urethral and cervical swab specimens sent to a clinical microbiological department to be examined for gonococci

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MATERIALS AND METHODS

Urethral and Cervical Swab Specimens

The material consisted of urethral (male or female) and cervical swab specimens collected between 10th and 20th January 1974 and sent to the Department of Medical Microbiology University Hospital, Lund, Sweden, for cultivation for gonococci. The specimens were collected with a sterile cotton swab impregnated with charcoal (9) and sent to the Department in modified Stuart transport medium (16) within 24 hours of collection. Cultures from 364 female patients were examined. 42 of these patients came from departments of venerology as did 78 of the 162 male patients.

Examination for N gonorrhoeae

Gonococci were isolated and identified by fermentation tests as described by *Lind* (12) except that the antibiotics, polymyxin B (Novo) 25 I.U and trimethoprim (Roche) 3 μg/ml (see *Ödegaard* (15)) were added to the isolation medium.

Examination for Streptococci

The swabs were streaked onto 5 per cent horse sheep blood agar plates (10) The plates were incubated anaerobically (BBL Gas-Pac system) over night at 37° C. Beta-hemolytic streptococci and non-hemolytic enterococci were serologically grouped as described previously (6) with the use of rabbit specific antibodies to Lancefield group A, B, C, D and G adsorbed to protein A containing staphylococci. The identity of group A streptococci was also checked with the "Tripple plate test" (18) and the group B streptococci with the sodium hippurate test (1) A 100 per cent correlation was found between biochemical and serological grouping methods. All the group D streptococci produced black colonies on the Mcleod medium containing potassium tellurite in a concentration of 1 2500 and thus belonged to the *Streptococcus faecalis* group (17)

RESULTS

Frequencies of Beta-hemolytic Streptococci and N gonorrhoeae in Urethral and Cervical Swab Specimens from Patients with Suspected Genital Gonococcal Infection

In all, specimens from 526 patients, 364 females and 162 males were examined (Table 1) 22.3 per cent of the females harboured group B streptococci in the urethra and/or cervix. The frequency of group D streptococci was 17 6 per cent. Group C streptococci were isolated from 3 female patients group G from 2. Group A were not encountered.

The frequency of group B and D streptococci in the urethra of the male patients were 11 7 and 9.3 per cent, respectively Group A streptococci were isolated from 3 patients, group C from 1 and group G from 1

Eight of the females had group B as well as D streptococci 1 female had group B, D and G streptococci, and 1 female had C and D

Groups B and D streptococci did not vary significantly in frequency with the patients ages.

The overall frequencies of beta-hemolytic streptococci were 0 6 per cent group A, 19.0 per cent B, 0.8 per cent C, 15.0 per cent D and 0.6 per cent G

TABLE 1 *Frequencies of Beta-hemolytic Streptococci and N gonorrhoeae in Urethral and Cervical Swab Specimens from Patients with Suspected Gonococcal Infection*

Sex	Number of patients	Number of patients with beta-hemolytic streptococci					Number of patients with *N gonorrhoeae*
		Group A	Group B	Group C	Group D	Group G	
Females	364	0 (0 0 %)	81 (22.3 %)	3 (0.8 %)	64 (17.6 %)	2 (0.5 %)	20 (5.5 %)
Males	162	3 (1.9 %)	19 (11 7 %)	1 (0.6 %)	15 (9.3 %)	1 (0.6 %)	21 (13.0 %)
Total	526	3 (0 6 %)	100 (19.0 %)	4 (0.8 %)	79 (15.0 %)	3 (0.6 %)	41 (7.8 %)

8 females had both group B and D streptococci. One female had B, D and G streptococci and one had C and D

TABLE 2. *Comparison between Specimens from Patients from Departments of Venereal Diseases and from Other Patients*

Sex	Department	Total number of patients	Number of patients with group B streptococci	Number of patients with group D streptococci	Number of patients with N gonorrhoeae
Females	Departments of venereal diseases	42	15 (35.7 %)	4 (9.5 %)	4 (9.5 %)
	Other departments or general practitioners	322	66 (20.5 %)	60 (18.6 %)	16 (5.0 %)
Males	Departments of venereal diseases	78	10 (12.8 %)	4 (5.1 %)	9 (11.5 %)
	Other departments or general practitioners	84	9 (10.7 %)	11 (13.1 %)	12 (14.3 %)

N gonorrhoeae was demonstrated in 7.8 per cent of the 526 patients (in 5.5 per cent of the females and in 13.0 per cent of the males)

Occurrence of Group B and D Streptococci in Urethral and Cervical Swab Specimens from Patients with N gonorrhoeae

Nine (45.0 per cent) of 20 females with *N gonorrhoeae* and 4 (19.0 per cent) of 21 males harboured *group B streptococci in all* 31.7 per cent of 41 patients, which was higher ($p < 0.05$) than the frequency of group B streptococci in patients without gonococcal infection. None of the patients had *group D streptococci*. This was lower ($p < 0.01$) than the frequency of group D streptococci in patients without gonococcal infection.

Comparison between Specimens from Departments of Venerology and from Other Departments

Of 42 female patients from departments of venerology 35.7 per cent harboured group B and 9.5 per cent group D streptococci, while gonococci were found in 4 (9.5 per cent) patients (Table 2) In specimens from 322 patients from other departments or general practitioners the frequencies were 20.5 per cent, 18.6 per cent and 5.0 per cent, respectively The frequency of group B streptococci isolated from female patients from departments of venerology was higher ($p < 0.05$) than from the remaining patients.

Specimens from 78 male patients were from departments of venerology 84 from other departments or general practitioners. The frequencies of group B streptococci were

TABLE 3 *Isolation of Group B and D Streptococci and* N gonorrhoeae *from Urethra and Cervix in Females*

Bacteria	Patients from whom bacteria were isolated	Number of isolates: From both urethra and cervix	From urethra only	From cervix only
Streptococci group B	81	37 (45.7 %)	39 (48.1 %)	5 (6.2 %)
Streptococci group D	64	34 (53.1 %)	23 (35.9 %)	7 (10.9 %)
Neisseria gonorrhoeae	18	8 (44.4 %)	1 (5.6 %)	9 (50.0 %)

* Values in bracket denote percentage of the total number of patients showing growth of respective bacteria in urethra and/or cervix.

respectively 12.8 per cent and 10 7 per cent group D 5 1 and 13 1 per cent and gonococci, 11.5 and 14.3 per cent.

The Frequencies of Group B and D Streptococci and N *gonorrhoeae in Patients with Suspected Gonococcal Infection Comparison between Frequencies in Urethral and Cervical Swab Specimens*

Of 81 females with group B streptococci, 45 7 per cent harboured group B streptococci in both the urethra and the cervix, 48.1 per cent only in the urethra and 6.2 per cent only in the cervix (Table 3) The frequencies for 64 female patients with group D streptococci were 53 1 35.9 and 10.9 per cent, respectively and for *N gonorrhoeae* 44 4 5.6 and 50.0 per cent, respectively (18 patients from 2 of 20 females with gonococcal infection were only cervical swab specimens obtained)

DISCUSSION

The frequency of group B streptococci (22.3 per cent) in the females was considerably higher than the figures given by other authors. It it quite possible that even higher frequencies could be found if selective media were used the isolation media used for appraising the frequencies of streptococci of groups A, B, C D and G in this investigation are not selective. *Hood et al.* (8) reported that 5.8 per cent of women at term (118 patients) harboured group B streptococci in the cervix. *Bergqvist et al.* (5) found such streptococci in 14 per cent of 118 pregnant women at term (urethral and cervical swab specimens) *Lancefield & Hare* (11) found 26 (3 per cent) of 837 clinically uninfected women to have group B streptococci (cervical swab specimens) during the puerperium. The higher frequency found in the present investigation among patients with suspect gonococcal infection lends support to the view that group B streptococci, like gonococci, in women are spread by sexual intercourse.

The epidemiology of group B streptococci in the female genital tract is also elucidated by other findings in this investigation 13 (31 7 per cent) of 41 patients of both sexes with gonococcal infection harboured group B streptococci, compared with 87 (18.8 per cent) of 481 patients without genital gonococcal infection. Furthermore, 15 (35 7 per cent) of 42 females from whom material was collected at departments of venerology harboured group B streptococci, compared with 66 (20.5 per cent) of 322 female patients from other departments or general practitioners.

On the other hand, no such correlation was found for group D streptococci. None of the patients with gonococcal infection harboured group D streptococci and thereby differed significantly from the patients without genital gonococcal infection.

The frequency of group B and D streptococci in the urethra in the males was notably higher than that found in other investigations. *Meyer Rohn* (14) found *Streptococcus pyogenes* (no grouping performed) in 6 out of 100 normal males. *Streptococcus faecalis* was found in 2 per cent. *Furness et al.* (7) found alpha- and beta-hemolytic streptococci (not further specified) in 23 per cent of normal controls and in 31 per cent in males with nonspecific urethritis.

No significant difference in frequencies of group B and D streptococci was found between the males from departments of venerology and other males. The females from the departments of venerology constituted a more highly selected group 42 of 364 came from such departments compared with 78 of 162 males (compare also the frequencies of *N gonorrhoeae* Table 2)

Furthermore, the investigation showed that group B and group D streptococci were found more often in the female urethra than in the cervix. Screening of women for group B streptococci should therefore always include urethral swab specimens.

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QUANTITATION OF THE UPTAKE OF HUMAN IgG BY SOME STREPTOCOCCI GROUPS A, B, C AND G

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The uptake of IgG by streptococci irrespective of the antibody combining sites was studied with a method using [125]I labelled pooled human IgG and human IgG myeloma proteins. When 1 μg IgG was added it proved possible to quantitate the uptake of IgG within a standard deviation of 3 per cent. The results expressed relative to the 1.0 μg IgG added were: group A, type M 56: 20.4±1.4 per cent, group B, strain 123 B: 40.8±1.6 per cent, group C, strain 81 C: 44.9±1.9 per cent and group G, strain 115 G: 47.0±2.8 per cent. When IgG was added in excess, two of the streptococcal strains, group A, type M 1 and group G, strain 115 G were able to take up about one third of that bound by a similar number of the *S. aureus* Cowan I. Only minor differences were seen between the uptake by the streptococci of a given amount of four randomly selected purified myeloma proteins of IgG subclasses 1, 2, 3 and 4 and pooled human IgG with the exception of a group G strain which took up a smaller amount of IgG 3 myeloma protein. Trypsin treatment and heating of the streptococci at 100° C for 15 min reduced the uptake of IgG by streptococci to a certain extent while heating of the streptococci at 56° C for 30 min did not alter the uptake of IgG. Lancefield extract of group A, type M 56 streptococcus inhibited such uptake of IgG.

By interaction between immunoglobulins and streptococci (*Kronvall* 1973) some streptococci of group A, B, C, D and G (*Christensen* & *Kronvall* 1974) co-agglutinate sheep red cells coated with a sub-agglutinating dose of rabbit anti-sheep red cell antibodies. No agglutination of non-sensitized sheep red cells occurred. It was not possible to estimate the sensitized sheep red cell-agglutinating capacity on the surface of the individual streptococcal strain because of steric inhibition.

The uptake of [125]I labelled purified myeloma proteins and pooled human IgG is measured in the present investigation. Protein A from *Staphylococcus aureus* reacts with the Fc portion of IgG; a similar method is available for quantitation of the number of protein A residues (*Kronvall et al.* 1970). In this method the bacteria are allowed to react with a known amount of isotope labelled IgG after which the uptake is quantitated by centrifugation of the suspension followed by measurement of radioactivity in the sediment.

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MATERIALS AND METHODS

Bacterial Strains

Streptococcus group A, type M 56 (No. 100191) was kindly supplied by the Streptococcal Depart

ment, Statens Seruminstitut, Copenhagen, and type M 1 (No. 8198) and M 5 (No. 100065) by the Central Public Health Laboratory London. The group B C and G strains used in the experiments were obtained from routine bacteriological specimens. *Grouping of streptococci was performed with* staphylococci coated with specific antibodies by means of the protein A—Fc reaction (*Christensen et al.* 1973)

The *Staphylococcus aureus* strain Wood 46 known not to produce protein A was heated and treated with formalin in the way described by *Arowall* (1973) Addition of Wood 46 kept the volume of organisms after centrifugation constant in experiments measuring the uptake of IgG by different amounts of streptococci. The *S aureus*—strain, Cowan I (NCTC no. 8530) producing protein A, treated in a similar way and 5 strains of *E. coli* 5 *H influenzae* and 4 *S albus* strains served as controls.

Densimetry

The streptococci were quantitated by measuring the optical density at 540 μ, which reflects the weight of bacteria more than their number (*Mallette* 1969) *Care was taken to measure the optical* density of such streptococci that were grown under identical conditions, i.e. all streptococci were cultured for 18 hours on 10 ml Todd Hewitt broth inoculated with roughly the same amount of streptococci. Strains, which showed excessive spontaneous agglutination were not used in the study. The streptococci were washed 3 times in phosphate buffered saline (PBS 0.12 M NaCl, 0.03 M phosphate, pH 7.2) and suspended in PBS to an extinction of 0.37 in a Linson 3 photometer. By centrifugation, the bacteria were concentrated 10-fold (corresponding to about 2.5×10 bacteria/ml) This concentration was used as a stock suspension for all streptococcal strains in the experiments.

The *E. coli*, *H influenzae* and *S albus* strains used as controls were counted in a Petroff Hauser chamber and suspended to 2.5×10^{1} bacteria/ml.

Myeloma Proteins

IgG proteins of all 4 IgG heavy chain subclasses were isolated from sera of patients with multiple myeloma by the use of methods as agarose gel electrophoresis (*Laurell* 1965) DEAE chromatography (*Peterson & Sober* 1956) and Sephadex G 200 gel filtration (*Flodin & Killander* 1962) The isolated proteins (2.5 mg/ml) were not contaminated with other subclasses tested by immunoelectrophoresis (*Scheidegger* 1955) with IgG subclass specific antisera (*Oxelius* in press)

Human Pooled IgG

Commercial pooled human IgG was purchased from AB Kabi (batch no. 44791)

Protein Determination

The protein was measured with a modification of Folin's method (*Lowry et al.* 1951)

Radioiodination of Immunoglobulins

Commercial pooled human IgG and the isolated myeloma proteins were labelled with ^{125}I according to *McConahey & Dixon* (1966) To minimize aggregation the preparations were dialysed against a 0.05 M phosphate buffer pH 6.8, containing 0.5 M NaCl and 0.1 M glycin (*Hanson* 1968) Before use the labelled IgG was centrifuged at 5,000 × *g* for 1 hour to remove grossly aggregated proteins.

Determination of the Uptake of IgG by Streptococci

Isotope labelled IgG (in a volume of 50 μl) was incubated with the streptococci suspended in 200 μl PBS (varying time, *temperature* and amount of streptococci) After addition of 2 ml PBS containing 0.05 per cent Tween 20 the suspension was centrifuged at 3000 × *g*, for 30 minutes. The clear supernatant was aspirated and the radioactivity of the sediment was measured in a gamma scintillation counter (Selektronik, Hornbolm, Denmark)

It is well known that proteins adhere to glass and plastic materials (*Sutherland & Campbell* 1958) The amount of protein adhering to the tubes varies inversely with the amount of IgG deposited. When 65 μg, 6.5 μg and 0.65 μg ^{125}I labelled IgG (pooled commercial IgG or IgG 1 myeloma protein) were incubated with 200 μl PBS for 1 hour in the plastic tubes used in the experiments (70 × 11 mm tubes, A/S Nunc, Roskilde Denmark) and centrifuged after addition of 2 ml PBS containing 0.05 per cent Tween 20, the amounts of IgG adherent to the tube walls were 3 10 and 20 per cent, respectively of the IgG added. The amount of IgG adherent to the *test tube wall, the radioactivity* bound in the bacterial sediment fluid and the radioactive background were estimated by running a control in every experiment with an *E. coli* strain. Before calculating the streptococcal uptake *the* total radioactivity count in the control was subtracted from the values obtained with the streptococci. The pellets from 5×10^{9} of the *E. coli* and four other *E. coli*, 5 *H influenzae* and 4 *S albus* contained 2.0 ± 0.7 per cent, 1.8 ± 0.2 per cent, and 1.8 ± 0.5 per cent respectively of 1.0 μg IgG added in addition to the amount adherent to *the* test tube walls: 17.0 ± 1.0 per cent.

Streptococcal Extracts

Streptococcal extracts were prepared as described previously (*Christensen & Kronvall* 1974)

with HCl and NaOH. The sediment from streptococci grown over night on 20 ml Todd Hewitt broth was suspended in 0.15 ml 0.2 N HCl, heated for 10 min at 100° C and neutralized with 0.45 ml 0.5 N Na_2HPO_4. A similar amount of streptococci was suspended in 0.5 ml 0.2 N NaOH heated for 1 hour at 100° C and neutralized with 0.5 ml 0.1 M acetic buffer After centrifugation, the supernatants were used as extracts.

Trypsin Treatment of Streptococci

1.5 ml streptococci (stock suspension) was washed once in 0.1 M tris (hydroxymethyl) aminomethane (Tris) pH 8 0 and then suspended in 1.5 ml Tris. Trypsin (Sigma) 1 mg was added. An aliquot of 200 µl streptococci was taken out of the suspension after 5 15 30 and 60 min at 37 C. A 1 per cent solution of a Soya bean trypsin inhibitor (Sigma) 0.1 ml was added, after which the mixture was washed three times in Tris, resuspended in 200 µl Tris and incubated at 37 C again. In this way all aliquots were incubated for 1 hour After washings in PBS, the uptake of isotope labelled IgG was determined. The streptococci were also treated 18 hours with trypsin. The control bacteria were treated in the same way but without trypsin.

Heat Treatment of Streptococci

200 µl of each stock suspension of group A, B, C or G strain were incubated for 30 min at 37 C and 56° C and 15 mm at 100° C The uptake of isotope labelled IgG was then measured.

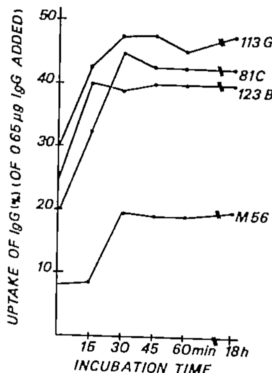


Fig 1 The influence of incubation time on the uptake of [125]I labelled IgG-1 myeloma protein by streptococci. 0.2 ml stock suspension of group A, type M 56 group B, strain 123 B group C, strain 81 C or group G strain 113 G was incubated t room temperature with 1 0 µg IgG-1 the uptake of IgG then determined.

RESULTS

1 Standardization of the Experimental Conditions

Incubation temperature The uptake of [125]I labelled pooled IgG or IgG 1 myeloma protein (1 µg added) by one strain of each groups A, B, C or G were tested at 0 20 37 and 45 C. The uptake by the strain did not vary markedly with the four temperatures used.

Incubation time The uptake of isotope labelled human pooled IgG and IgG 1 myeloma protein (1 µg) by 200 µl stock suspension of one strain of each groups A, B, C or G were measured after incubation for 0, 15 30 45 and 60 min and 18 hours at 20 C (Zero time indicates that centrifugation was initiated immediately after admixing IgG and streptococci)

Fig. 1 gives the results obtained with IgG 1 myeloma protein. Similar results were obtained with pooled human IgG No further uptake was observed after 30 mm (Fig 1) In the quantitative method the suspension was incubated for 60 mm before centrifugation.

Separation of bacteria and fluid phase After incubation of 200 µl stock suspension of one strain of each groups A, B, C or G with 1 µg labelled IgG 1 myeloma protein or 1 µg human pooled IgG for 1 hour at 20 C, 2 ml of PBS containing 0 05 per cent Tween 20 was added to the test sample before centrifugation to eliminate the radioactivity in the fluid included in the bacterial sediment. Four additional washings eluted 0 7 1 4 2.0 and 0 8 per cent (mean values for the four strains tested) of the IgG 1 myeloma protein

and 0.5 1 4 2.0 and 1 7 per cent of the human pooled IgG added. In subsequent studies the radioactivity was counted after one washing.

Standard deviation of the values for the uptake of IgG by streptococci The uptake of IgG 1 myeloma protein by 200 µl stock suspension was measured for one strain of each groups A, B, C or G cultured in each 10 samples of Todd Hewitt broth. The results expressed in percent of the 1 0 µg IgG added were group A, type M 56 20 4±1 4 per cent group B, strain 123 B 40.8±1 6 per cent, group C, strain 81 C 44.9±1.9 per cent and group G strain 113 G 47 0±2.8 per cent.

Influence of varying the amount of streptococci 0 65 µg IgG 1 myeloma protein was added to increasing volumes of the stock suspensions of streptococci of group A, B C or G (the protein A negative strain Wood 46 was added to keep the volume of the sediment constant the streptococci and the *S aureus* were suspended in 200 µl PBS)

The results obtained are given in Fig 2 Similar results were obtained with the pooled isotope labelled IgG The inclination of the curve for uptake of IgG by the strain 123 B is steeper than that of the curves for the

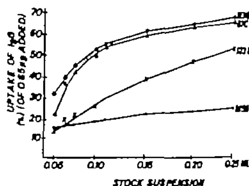


Fig 2 Uptake of [125]I labelled IgG-1 myeloma protein by increasing volumes of the stock suspensions of group A, type M 56, group B, strain 123 B, group C, strain 81 or group G, strain 113 G. The protein A negative *S aureus* strain, Wood 46, was added to keep the volume of the sediment constant.

group A, C and G streptococci. An increase in the volume of the stock suspension above 100 µl caused relatively little further increase in the uptake of IgG by the streptococci group A, C and G

Uptake of IgG by streptococci incubated with increasing amounts of IgG The uptake of [125]I labelled human pooled IgG by 200 µl of the stock suspensions of streptococci of group A, B C or G incubated with 1.0, 6.5, 65 0 or 250 µg IgG is shown in Table 1 The

TABLE 1 *Uptake of [125]I Labelled Pooled Human IgG by Streptococci (200 µl Stock Suspension) Incubated with Varying Amounts of IgG*

Streptococcal group	Strain	Amounts of IgG added			
		1 µg	6.5 µg	65 µg	250 µg
A	type M 1	0.56*	4.3	13.0	13.5
	type M 5	0 20	1 4	9 4	8 5
	type M 56	0 20	1 1	6.9	7 0
B	123 B	0 40	1.3	2.5	3 0
	256 B	0.04	– †		–
	271 B	0 02	–		–
C	81 C	0.45	2 9	6 5	8 0
	273 C	0.20	0.4	1 7	2 5
	281 C	0.20	0 7	8 4	7.5
G	*113 G*	*0 50*	3.3	14 3	12 5
	117 G	0.43	2.7	5.5	6 0

* The values are given in µg

† Not measurable

TABLE 2. *The Uptake of IgG—Myeloma Proteins and Pooled IgG by Streptococci*

Streptococcal group	Strain	Uptake of isotope labelled IgG by 200 µl stock suspension of streptococci in per cent of 1.0 µg IgG added				
		IgG-myeloma proteins, subclass				Pooled IgG
		1	2	3	4	
A	type M 1	50	55	40	74	56
	type M 5	25	20	11	25	20
	type M 56	20	15	12	21	20
B	123 B	40	34	41	40	40
	256 B	1	9	4	9	4
	271 B	4	9	6	4	2
C	81 C	48	62	41	59	45
	275 C	23	39	37	29	20
	281 C	12	21	15	15	20
G	113 G	57	65	19	60	50
	117 G	48	40	4	63	53
	147 G	4	11	7	6	4
S aureus Cowan I (5×10^8 bacteria)		95	90	1.5	95	92

uptake of IgG when 250 µg or 65 µg IgG was added did not differ significantly from each other which showed a maximal uptake of IgG by 200 µl stock suspension of the streptococcus group A, type M1 and the group G strain 113 G of 13.0–13.5 and 12.5–14.5 µg IgG respectively. The uptake of IgG by 5×10^8 Cowan I staphylococci was 30 µg IgG when 150 µg IgG was added. The stock suspensions, 200 ml of streptococcus group A, type M1 and group G strain 113 G bound 12.5 µg and 13.5 µg, respectively of 65 µg ^{125}I labelled IgG 1 myeloma protein.

The values for the uptake from 1 µg IgG by 200 µl stock suspension of streptococci differentiate between strains with high and low maximal uptake of IgG such as between M 1 and 113 G on one hand, and 256 B and 271 B on the other hand. However the uptake of IgG by streptococcus group A, type M 56 and group B, strain 123 B was 0.20 µg and 0.40 µg when 1.0 µg IgG was added, respectively but the group A strain took up more of the IgG added in excess than did the group B strain.

2 Role Played by Antibody Combining Sites in Uptake of IgG by Streptococci

Uptake of IgG myeloma proteins of subclasses 1 2 3 and 4 by the selected streptococci. The probability that the antibody combining sites of four randomly selected IgG—myeloma proteins would all react with streptococcal antigens is negligible. 1.0 µg IgG myeloma proteins of subclass 1 2, 3 or 4 were added to 200 µl of the stock suspensions of streptococci of groups A, B C and G The uptake of myeloma proteins and human pooled IgG by the individual strain was similar with the exception, for example, of the smaller uptake of IgG 3—myeloma protein by strain 113 G (Table 2) Apart from this, the variation of the uptake of subclasses by the individual strain reflects probably only minor differences in the affinity for the four subclasses.

3 The Reaction of some Streptococcal Preparations with IgG

Influence of trypsin treatment. The uptake of isotope labelled IgG 1 myeloma protein by one group A, one group B and one group

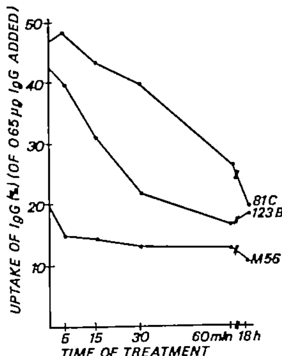


Fig 3 The influence of trypsin treatment of bacteria on the uptake of ^{125}I labelled IgG-1 myeloma protein by 0.2 ml stock suspension of group A, type M 56, group B, strain 123 B or group C, strain 81 C.

C strain diminished when the bacteria were treated with trypsin (Fig 3) After 18 hours' trypsin treatment the uptake diminished from 20 to 11 per cent, 42 to 18 per cent and 47 to 21 per cent of 1 µg IgG added for the group A, B and C strains, respectively For comparison the capacity of *S aureus*, Cowan I to bind IgG 1 myeloma protein diminished from 91 to 9 per cent of 1 µg IgG added after trypsin treatment for 1 hour this is in accord with earlier findings (*Lind & Mansa* 1968)

Effect of heat treatment. The uptake of myeloma IgG by the streptococci was practically unchanged after heating the bacteria for 30 min at 20 37 and 56° C while the uptake diminished to some extent after heating at 100 C for 15 min (Table 3)

Inhibition experiments with extract from streptococci group A B C and G The uptake of ^{125}I labelled IgG 1 myeloma protein by 200 µl of the stock suspension of the group B, strain 123 B was measured before and after addition of hydrochloric acid or

TABLE 3 *The Effect of Heat Treatment of Streptococci on the Uptake of IgG by Streptococci*

Heat treatment		Uptake of isotope labelled IgG-1 myeloma protein in per cent of 1 µg added			
Temperature	Time of treatment	Group A, type M 56	Group B, strain 123 B	Group C, strain 81 C	Group G strain 115 G
20 C	30 min	21	41	46	49
37 C	30 min	20	41	47	49
56 C	30 min	15	33	46	48
100 C	15 min	15	19	30	57

TABLE 4 *Effect of Extracts of Streptococci on the Uptake of IgG by Streptococci*

	Before adding extract	After adding extracts							
		Group A extract		Group B extract		Group C extract		Group G extract	
		HCl	NaOH	HCl	NaOH	HCl	NaOH	HCl	NaOH
Uptake of IgG-1 myeloma protein by group B streptococci in per cent of 1 µg added	40 %	27 %	26 %	36 %	27 %	41 %	23 %	41 %	[illegible]

* 200 µl stock suspension of group B streptococci were allowed to react with 1 µg IgG-1 myeloma protein before and after adding neutralised HCl—and NaOH extracts of streptococci group A, B, C and G.

sodium hydroxide extracts of the streptococci (Table 4)

The highest inhibitory activity was found in the NaOH extract. The HCl extract from M 56 as well as from the group B streptococcus showed some inhibition, while the HCl extract from the group C and G streptococci did not show any measurable activity

Control experiments with 50 μl of neutralized HCL and NaOH showed no inhibitory effect.

DISCUSSION

Several reports indicate the existence of a structure in some streptococci reacting with IgG irrespective of the antibody combining sites. Group A, C and G streptococci have been reported to show fluorescence with FITC-labelled preimmune rabbit IgG (*Danielsson* 1965 *Jonas & Foster* 1966 and *Lind* 1967) The ability of some group A, B, C, D and G streptococci to agglutinate sheep red cells coated with a sub-agglutinating dose of rabbit-anti-sheep red cell antibodies, but not non-sensitized sheep red cells (*Kronvall* 1973 *Christensen & Kronvall* 1974) indicates a binding between the streptococci and the rabbit immunoglobulin which has its antibody combining sites attached to the sheep red cell antigens. The inhibition of uptake by streptococci of ^{125}I labelled human pooled IgG by myeloma sera correlates with the IgG-myeloma protein content in the sera, but no inhibition is seen with Fab fragments of IgG (*Kronvall* 1973)

Other authors have produced indirect evidence for the existence of such a reactivity *McIntosh et al.* (1971 1972) reported that nephritogenic streptococci altered the chemical composition and the immunogenicity of IgG either by a simple mechanism or by interaction of the immunoglobulin with a protein from the organism, as, e.g. between gamma-globulins and protein A from *Staphylococcus aureus*"

In a work about a toxic moiety in M—protein showing "almost universal reactivity" with the platelets and polymorphonuclear leucocytes of normal human blood, *Stollerman* (1972) remarked "The question of whether the M—associated toxic factor represents a substance similar to the protein A of staphylococci remains to be determined."

The interaction between IgG and streptococci was studied further in this investigation with a method using ^{125}I labelled human pooled IgG and IgG myeloma proteins. By the use of purified myeloma proteins, the probability of participation of the antibody combining sites in the reaction was minimized the fact that commercial pooled human IgG and IgG myeloma proteins of the 4 subclasses were taken up in similar amounts strengthen the assumption that the main part of the uptake of IgG by the streptococci tested here is independent of the antibody combining sites. Major differences in the reactivity with IgG subclasses as, e.g., the failing reactivity of protein A from *S aureus* with IgG 3 (*Kronvall & Williams* 1969) were not found

The uptake of isotope labelled IgG by streptococci could be determined within a standard deviation of 5 per cent of 1 μg IgG added. The streptococci reached a maximal level of uptake of IgG after incubation with the IgG for 30 min at room temperature. Two of the streptococcal strains, group A, type M 1 and group G strain 113 G were able to take up about 13 μg IgG when IgG was added in excess to 200 μl stock suspension of the streptococci. The capacity of a similar number of *S aureus* Cowan I to take up IgG under similar conditions was 50 μg IgG The uptake of IgG by different strains within the same groups, A, B, C or G showed remarkable differences between the strains. The biological significance, if any of this finding is receiving attention.

An interaction between IgG and some preparations of streptococci commonly applied in serological grouping and typing of streptococci was shown. The investigation showed that the non-specific, antibody combining sites independent reaction between streptococci and IgG does not disappear after trypsin treatment but diminishes. Trypsin treat

ment is used in grouping by agglutination in group specific sera (*Rosendal* 1956) and T—typing of streptococci (*Griffith* 1934) to homogenize spontaneous agglutinating streptococci and to remove the M protein.

Non-specific reactivity between streptococci and IgG was demonstrated also in hydrochloric acid extracts of streptococcus group A, type M 56 indicating that this reactivity might perhaps take part in the precipitation technique for M—typing of this strain. The streptococcal grouping and typing sera are often absorbed with heat treated streptococci to prevent cross reactivities. The finding in this investigation that heating up to 100 C for 15 min does not remove the structure on the streptococci reacting with IgG irrespective of antibody combining sites, explain the diminishing IgG content in, for example M—typing sera when they are absorbed by streptococci (See *Rotta* 1972) It would appear that a reappraisal of the streptococcal serological typing systems is called for

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INFLUENCE OF OSMOTIC PRESSURE ON TRANSFORMABLE AND NON TRANSFORMABLE VARIANTS OF *NEISSERIA MENINGITIDIS*

1 *Survival and Turbidity Changes*

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The reactions of competent (*cp*) and incompetent (*cp*) variants of the *Neisseria meningitidis* Strain M1 to variations in the osmotic pressure have been compared by observing survival and absorbancy changes. Pronounced differences were observed. Both competence variants were very sensitive to osmotic pressures outside a range between approximately 4 and 7 atmospheres, but the *cp* cell had a much higher survival when exposed both to increased tonicity and to hypotonic conditions. With the assumptions valid for smaller systems, the undamaged *N meningitidis* cells from both competence variants behaved as osmometers at least between osmotic pressures from around 1.5 atmospheres to above 24 atmospheres. Exposure of the cells to distilled water as well as to high osmolality however, interfered strongly with the capacity of the cells to maintain an osmotic barrier suggesting membrane damage. This impairment was far more pronounced in *cp* cells than in *cp* ones, indicating that the cytoplasmic membrane of the *cp* cell is relatively protected against deleterious effects of osmotic stress. Some implications of these findings have been discussed

Several differences have been observed between the phenotypes of the genetically competent (*cp*) and incompetent (*cp*) variants of the *Neisseria meningitidis* Strain M1 These differences include sensitivity to the chemicals nitrosoguanidine (12) ethidium bromide and acriflavin (13) as well as presence of surface structures such as fimbriae (5) The present study was undertaken to explore the idea that a main difference might lie in the realm of the membrane structure or function. The experimentation leans strongly on the phenomenon of absorbancy changes upon variations in osmotic pressure to demonstrate any functional differences in the cytoplasmic membranes

The absorbancy of various Gram-negative bacteria is affected by the tonicity of the suspending medium. Since both electrolytes and non-electrolytes cause turbidity changes, the optical effect seems to be essentially an osmotic phenomenon. An increase in absorbancy observed when cells are exposed to increasing tonicities has usually been ascribed to cell shrinkage (2, 19) or in some instances to the substrate becoming associated with the cell membrane (23) A decrease in absorbancy which is often observed has been attributed to swelling accompanying substrate uptake (1 22 27)

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When Gram-negative cells are placed in hypertonic medium, plasmolysis occurs. Loss of internal water causes the cytoplasm to shrink, and the plasma membrane to retract from the cell wall. The process is a rapid one, but it is often only temporary and does not necessarily interfere with the ability of the cell to divide and form colonies afterwards. The presence of ions and nutrients reduces the prevalence of plasmolysis (4 24). It seems that the absorbancy of a plasmolysed cell suspension bears an inverse relationship to the cytoplasmic volume, and that the cytoplasmic component of the cell behaves as an osmometer at least at osmotic pressures above the treshold for plasmolysis (20 21). The values obtained from the plasmolysed cell suspension follow the van t Hoff Boyle law in a fashion analogous to those obtained from protoplasts (6).

When bacteria are placed in hypotonic solutions, plasmoptysis often occurs. In plasmoptysis the rigid cell wall is ruptured due to the expansion in cytoplasmic volume, and the internal contents of the bacteria are partially extruded through the breaks. This process is brought about by osmosis leading to an internal pressure sufficient to overcome the structural strength of the cell wall.

MATERIALS AND METHODS

Bacteria. The wild type *N. meningitidis* Strain M1 of Group B was used along with the auxotrophic mutants M1-6 *his pro* and M1-8 *his arg*. Variants which were competent in transformation were indicated by the symbol *cp* and incompetent ones by *p*. The growth requirements as well as competence in transformation were controlled as described previously (9 11).

Media and growth. Blood agar plates and Heart Infusion Broth (HIB Difco) agar plates were used as solid complete media. Fluid complete medium was HIB. The basal media were those used before (10). Growth was performed and followed by measuring absorbancy or colony-forming units as previously described (13 14).

Survival in hypertonic solutions. Unless otherwise stated, logarithmic phase cultures of *N. meningitidis* were used. HIB cultures were inoculated from a start culture in the exponential phase, and grown from A of about 0.100 to A close to 0.250 on a reciprocal shaker at 37 C. The cells were spun down (10 min at 2500 × *g*) washed once and resuspended in the lowest concentration of solute to be used in the actual experiment. Survival curves were obtained from equal amounts of cells exposed to hypertonic solutions under two different conditions 1) Exposure to sucrose or NaCl alone. 2) Exposure to sucrose or NaCl in the presence of the salts of the basal medium (K_2HPO 7 g KH_2PO_4 2 g NH Cl 1 g Na_2SO 0.05 g $MgCl_2$ 0.1 g $MnCl_2$ 0.001 g $CaCl_2 H_2O$ 0.05 g $Na_2S_2O_3$ 0.05 g and H_2O to 1 l). Counts were either performed directly from these suspensions, or after a period of incubation in a system corresponding to that used in transformation of *N. meningitidis* (8, 16). The system contained 1.7 ml HIB supplemented with 0.005 M $CaCl_2$ 0.2 ml cell suspension 0.1 ml NaCl-citrate buffer (0.15 M NaCl plus 0.015 M Na_3 citrate pH 7.5). Colony forming ability was determined after exposure as indicated in the individual experiments, by adding 0.1 ml of the suspension to 4.9 ml HIB. Further dilutions were carried out through HIB. Samples (0.1 ml) were plated in triplicate on complete agar plates. The plates were incubated at 37 C, and the colonies were counted when they became easily visible. This required incubation for 2 days at the higher concentrations of solutes.

Survival in hypotonic solutions. Cultures were inoculated and grown as described above. The cells were washed once either with 0.04 M phosphate buffer pH 7 containing 0.04 M $MgCl_2$, or with 0.15 M NaCl supplemented with 0.02 M $MgCl_2$. Equal volumes of cell suspension in the same solutions were spun down in the centrifuge in a number of small tubes, and the pellets were resuspended in distilled water or in the hypotonic solutions under study. After incubation at 20° C for the time indicated in the individual experiment, the exposure was interrupted by adding an equal volume of HIB of double concentration. Dilutions through HIB, and platings on complete agar plates followed the procedure described above.

Measurements of optical effects. The preparation of cell suspensions as well as the arrangement of the experiments followed the general procedures described above for the construction of survival curves. The test solutions were made on an osmolality basis, taking into consideration the activity coefficients of the various solutes, to obtain solutions of equal osmotic properties. Readings were made at room temperature at 600 nm in the Bauch & Lomb Spectronic 20 spectrophotometer. In some experiments the Beckman spectrophotometer model DB was used with 1 cm light path.

Correction for differences in refractive index. Two methods were used to eliminate the effects of the different refractive indices on the absorbancy. The first method followed the rationale of *Gilby & Few* (6). The refractive index of each medium was ascertained from the litterature (mainly from

Handbook of Chemistry and Physics, The Chemical Rubber Co., Cleveland Ohio 1967) and also checked with an Abbé "60" refractometer in many instances. The results from measurements of optical effects after exposure to increased tonicity (Fig. 5) were then used to plot, at fixed values of osmotic pressure the absorbancy as a function of Δ n, the difference in refractive index of the medium and water A straight line was drawn through the points and extrapolated to estimate the absorbancy of a refractive index equal to that of water Generally the calculations comprised pairs of absorbancy curves, so that finally all curves had been corrected against each other In the second method the masking effect on absorbancy by high refractive index was eliminated by the use of Ficoll according to *Myers et al.* (21) The sucrose-polymer Ficoll (*molecular weight approximately 400,000*) has little osmotic effect in concentrations giving a refractive index as high as 1.3700. *Ficoll correction* curves, prepared from *cp* and *cp* cells, always showed a drop-off in absorbancy value of the cell suspension with increasing refractive index due to Ficoll (Fig 7)

Quantitative test for the behaviour as osmometer When the membrane is impermeabel to an extracellular solute but fully permeable to the solvent, the volume changes produced by osmosis in a system exposed to media of different osmotic pressure should follow the van t Hoff-Boyle law (21)

$$\pi (V - V_o) = C \qquad (1)$$

Here π is the osmotic pressure of the medium, V the volume of the cell, V_o that volume of the cell which does not respond to osmotic influences, and C a constant. This law is followed by the cells of many higher organisms (6) If V = K/A where K is a constant and A the absorbancy of suspension (18) the equation may be re-written.

$$\frac{\pi}{A} = \pi \frac{V_o}{K} + \frac{C}{K} \qquad (2)$$

From this equation it follows, that provided the cells follow the van't Hoff Boyle law a plot of π/A against π should be linear

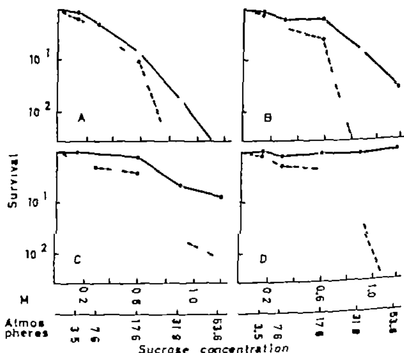


Fig 1 Survival of colony-forming ability in *N meningitidis* suspensions exposed to increasing concentrations of sucrose Comparison of *p* variant (solid lines) and *cp* variant (dotted lines) Cells in the exponential phase were exposed to the tonicity indicated for 20 minutes and counted as described in Methods. *Curve A* Exposure to sucrose only *Curve B Exposure to* sucrose in the presence of "basal salts *Curve C* Exposure to sucrose followed by incubation for 20 minutes in HIB plus $CaCl_2$ 0.005 M before dilution and plating *Curve D Exposure to* sucrose in the presence of "basal salts followed by incubation for 20 minutes in HIB plus $CaCl_2$ 0.005 M.

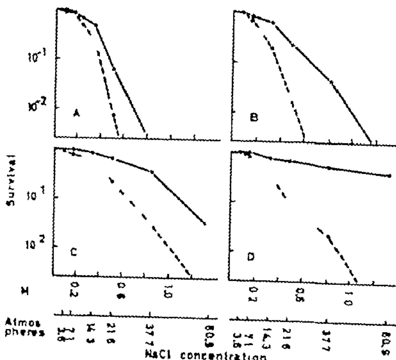


Fig. 2 Survival of colony-forming ability in *N. meningitidis* suspensions exposed to increasing concentrations of NaCl. Comparison of cp^+ variant (solid lines) and cp variant (dotted lines). Conditions as in Fig. 1. *Curve A.* Exposure to NaCl only. *Curve B.* Exposure to NaCl in the presence of "basal salts". *Curve C.* Exposure to NaCl followed by incubation for 20 minutes in HIB plus $CaCl_2$ 0.005 M before dilution and plating. *Curve D.* Exposure to NaCl in the presence of "basal salts" followed by incubation for 20 minutes in HIB plus $CaCl_2$ 0.005 M.

RESULTS

Survival of colony-forming ability in hypertonic solutions. Suspensions were first exposed to increasing tonicities above approximately 6 atmospheres osmotic pressure. Fig. 1 shows survival curves of exponential phase cells subjected to sucrose of various molarities. The survival was appreciably decreased as reported for other microbes under conditions conductive to more long lasting plasmolysis (25, 26). Similar results were obtained with NaCl (Fig. 2). The survival curves exhibit shoulders, indicating that the cells are relatively resistant at osmotic pressures up to around 7 atmospheres.

The experimentation showed clearly that the cp variant was far more sensitive to increased osmotic pressure than the cp^+ one under all conditions tested. The addition of salts ("basal medium salts", cf. Materials and Methods) reduced the lethal effect as observed in other bacteria (4, 24). Transfer to and incubation for 20 minutes in complete medium supplemented with 0.005 M $CaCl_2$ ("transformation system", cf. Materials and Methods) reduced the lethal effect further and more in the cp^+ than in the cp variant (8).

Survival of colony forming ability in hypotonic solutions. Fig. 3 shows survival curves of exponential phase cells subjected to decreasing concentrations of NaCl for 60 minutes. It is seen that the cells are relatively stable at osmotic pressures down to nearly 4 atmospheres, but at lower pressures a virtually linear killing is observed. But it is also seen that the cp cells are far more sensitive to hypotonic solutions than the cp^+ ones.

Curve A in Fig. 4 shows the survival of exponential phase *N. meningitidis* cells when

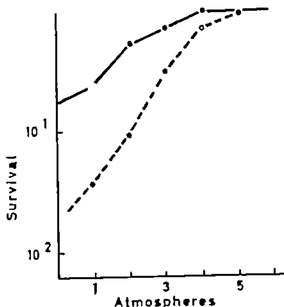


Fig 3 Survival of colony-forming ability in *N meningitidis* suspensions exposed to decreasing concentrations of NaCl Comparison of *cp* variant (solid lines) and *cp* variant (dotted lines) Cells in the exponential phase were exposed to the tonicity indicated for 60 minutes and counted as described in Methods.

exposed to distilled water for various times. It is seen that the cells are very sensitive to this treatment, and again it is observed that the *cp* variant is far more sensitive during prolonged exposure. But it is also seen that around 70 per cent of the cell population is very rapidly inactivated whereas the succeeding killing takes place more slowly This gives the *cp* variant a bipartite type of curve, a pattern which is less conspicuous in the *cp* variant.

Stationary phase cells (Curve B in Fig 4) are far more resistant than those from the exponential phase, and there is little difference between the over-all survival of the competence variants. But even in stationary phase cells the survival curve from the *cp* cells exhibits a bipartite pattern with a very rapid killing of around 30 per cent of the population.

Optical effects of tonicity variation. The *N meningitidis* suspensions exhibited pronounced optical effects when tested with a number of solutes, both electrolytes and non electrolytes. For a given solute concentration the percentage increase or decrease of absorbancy referred to the absorbancy in water as well as in a 0 04 M phosphate buffer supplemented with 0 05 M $MgCl_2$ (approximately 0.31 osmolal) remained constant over a wide range of dilutions (from A 0 050 to A above 0.600) in the Beckman spectrophotometer and was independent of the wave-

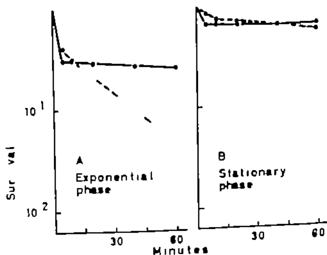


Fig 4 Survival of colony-forming ability in *N meningitidis* suspensions exposed to distilled water for various times. Comparison of *cp* variant (solid lines) and *p* variant (dotted lines) *Curve A* Cells from the exponential phase. *Curve B* Cells from the stationary phase.

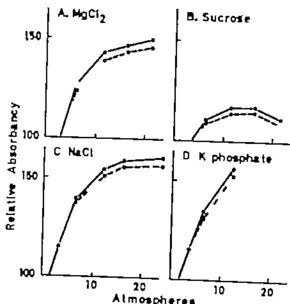


Fig. 5 Absorbancy of *N. meningitidis* cells from the exponential phase suspended in various solutions with increasing osmotic pressure. Solid lines *cp* variant. Dotted lines *cp* variant.

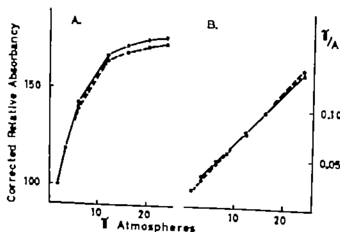


Fig. 6 Optical properties of *N. meningitidis* cells as a function of osmotic pressure. Solid lines: *cp* variant. Dotted lines *p* variant. The curves represent calculations based on data obtained with NaCl, sucrose, $MgCl_2$ and K-phosphate (Fig. 5). *Curve A.* Relative absorbancy corrected for refractive index of the medium. (First method, cf. Materials and Methods). *Curve B.* Plot of (Osmotic pressure)/(Absorbancy) against osmotic pressure. (Test of equation (2), cf. Materials and Methods)

length used. This is in general agreement with the findings from other microorganisms (19).

The experiments concerned with survival indicated that the *N. meningitidis* cells are easily damaged at osmotic pressures below around 4 atmospheres, and above approximately 7 atmospheres unless "basal salts" or complete medium are present. In order to reduce damage of the cells as much as possible, the bacteria were accordingly washed and resuspended in 0.27 osmolal of the solute to be tested in an actual experiment. Gene

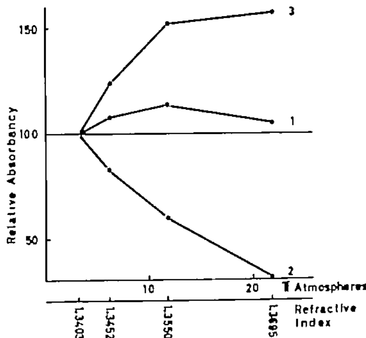


Fig 7 Absorbancy in a suspension of *cp* *N meningitidis* cells as a function of sucrose osmolality Correction for the refractive index of the med um by means of Ficoll. *C rve 1* The absorbancy measured. *C rve 2* Absorbancy in the presence of Ficoll with equal refracti e index. *Curve 3* Absorbancy corrected for the refractive index in sucrose

rally the capacity to cause absorbancy change was determined at increasing osmolalities up to around 1 4 osmolal.

The effects of sucrose, NaCl, MgCl and K phosphate were first compared in a number of experiments Fig 5 summarizes some of the results. In all instances increased osmotic pressure resulted in enhanced absorbancy at least from around 1.5 atmospheres upwards (Curve C and Curve D of Fig 5) But the most conspicuous finding was a systematic difference between *cp* and *cp* cells The increase in absorbancy was constantly less in suspensions of the latter type of cells under otherwise identical conditions.

In order to use these results to determine whether *N meningitidis* cells obey the van t Hoff Boyle law corrections were first made for the refractive index of the medium by the two procedures described in Materials and Methods. Graph A of Fig 6 shows the corrected curves based on experiments with sucrose, NaCl MgCl and K phosphate according to the first method (6) A Ficoll correction curve for cell suspensions in sucrose such as used in the second method (21) has been presented in Fig 7 The curves obtained by the second method were so similar to those presented in Fig 6 that no separate plot is warranted

The experiments were subsequently extended to suspensions in the following solutes: Glucose KCl, CaCl and Na-glutamate. They all gave results similar to those described for sucrose NaCl, MgCl, and K-phosphate.

In Graph B of Fig 6 osmotic pressure (π) has been plotted against π/A according to equation (2) (cf Materials and Methods) It became apparent from these calculations that the results fit the van t Hoff-Boyle law within the error of the experiments, and provided that the assumptions described are valid (6)

Change in the optical effects after exposure to decreased or increased tonicity. A number of experiments were next performed to find out whether the killing observed after exposure to decreased or increased tonicity might

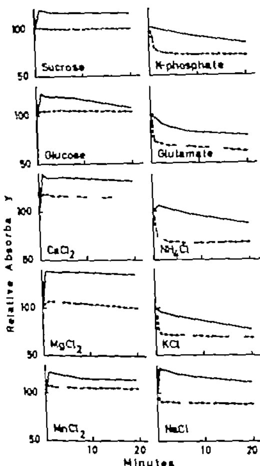


Fig. 8. Changes in the absorbancy of *N. meningitidis* suspensions caused by the addition of various solutes after pretreatment of the cells with distilled water. Solid lines: *cp* cells. Dotted lines: *cp* cells. The effect is given as A = (A at 6.5 atmospheres) /(A in distilled water) × 100 (19).

be connected with membrane damage. Presumably damage of the cytoplasmic membrane might cause permeability differences in the osmotic barrier which would modify the turbidity changes observed in undamaged cells.

Fig. 8 illustrates the absorbancy changes in suspensions of *N. meningitidis* cells from the exponential phase that have been pretreated by suspension in distilled water. It can be seen that the turbidity changes are very different from those observed in undamaged cells. The changes in absorbancy now differed from one solute to another and the difference between the competence variants became extremely pronounced. With sucrose, glucose, $CaCl_2$, $MgCl_2$ and $MnCl_2$, the absorbancy either still increased or remained the same. In all instances, however the increase was far more pronounced in the cp^+ variant. When K-phosphate, Na-glutamate, NH_4Cl, NaCl or KCl were used, a decline in absorbancy was regularly observed, but the decrease occurred much more rapidly in the *cp* variant. In the *cp* variant the decline in absorbancy tended to be preceded by a wave of increased absorbancy.

These patterns were on the whole reproducible, but the extent of increase or decrease in absorbancy differed somewhat from one experiment to another. Prolonged treatment with distilled water tended to reduce or totally abolish the absorbancy increase even in *cp* cells.

The effects of exposure to increased tonicity were next studied. In Fig. 9 the absorbancy increase in undamaged cells has been followed during two hours. It is seen that the initial rapid enhancement (relative to that at 3.5 atmospheres) tended to decrease upon prolonged incubation, and far more at higher than at lower osmolalities. At osmotic pressures below around 7 atmospheres, the increase in absorbancy tended to abide.

In a following series of experiments the cells were exposed to 24.5 atmospheres osmotic pressure for 30 minutes by either sucrose or NaCl. After the exposure, the cells were suspended at 3.5 atmospheres osmotic pressure. Subsequently the change in absorbancy upon increase in osmotic pressure to 6.5 atmospheres was followed. The turbidity changes now resembled those observed after exposure to distilled water (Fig. 8), indicating that the capacity of the cells to establish and maintain an increased absorbancy was drastically reduced, and far more in the *cp* than in the *cp* variant.

DISCUSSION

The optical density evidence from cells that are presumably undamaged, support the idea

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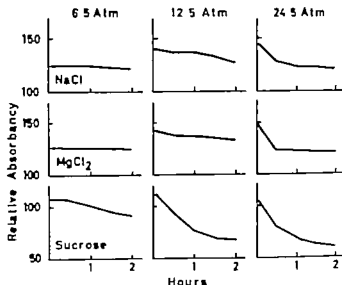


Fig 9 Absorbancy of *cp* *N meningitidis* cells from the exponential phase suspended in solutions of different osmotic pressure Changes during prolonged incubation at 20 C. Absorbancy is given relative to that at 3.5 atmospheres.

that the intact *N meningitidis* cells behave as osmometers upon changes in osmotic pressure at least from 1.5 atmospheres to above 24 Similar observations have been reported from other Gram-negative bacteria (20) and from Rickettsia (21) But the experiments also show that the absorbancy increase under otherwise identical conditions is somewhat more pronounced in *cp* cells. This may on the one hand indicate that the *cp* cell has an extra capacity for shrinkage (2 19) On the other hand, it may be connected with transport mechanisms. *Rogers* & *Yu* (23) observed absorbancy changes connected with the active permeation of substrate in *Escherichia coli*. They suggested that the effect was caused by an increase in material density at the cell membrane, due to the binding of the substrate in an osmotically inactive form. The authors drew an analogy between the concentration of substrate at the membrane, and the concentration of materials during aggregation processes. In such processes, absorbancy increases while material is being accumulated. It could thus be, that cp^+ cells have a higher capacity for substrate accumulation. Previous experiments have actually shown that the cp^+ cells in some instances may bind more material to the surface than the *cp* ones (17)

The survival curves show that osmotic pressure cannot be raised much above 7 atmospheres, or decreased below approximately 4 atmospheres without damage. Furthermore, the lethality connected with changes in osmolality was always less in the cp^+ variant. There are a number of observations supporting the notion that membrane damage plays an important part in this lethality.

In presumably intact *N meningitidis* cells the increase in absorbancy connected with osmotic pressures raised to around 7 atmospheres was a rather stable value. At higher osmotic pressures, however the absorbancy which was initially enhanced, started to decrease in less than 30 minutes. Such a comparatively slow decrease that usually follows the initial, rapid increase in absorbancy has been observed in other bacteria and animal cells, and has been ascribed to the gradually decreasing osmotic gradient as a result of the permeation of the solute (2, 18, 23). Swelling of the cells occur because water is taken up to compensate for the increased osmotic pressure in the cell (28). During exposure to hypertonic conditions, it seems that the

membranes become increasingly permeable to the solutes tested, and that the cells lose their capacity to maintain an osmotic barrier. In Gram-negative bacteria the exposure to osmotic pressures above 7–8 atmospheres is often connected with plasmolysis, and the spectrophotometric procedure has often been used as a measure of plasmolysis (21 26). It thus seems, that conditions that are generally conductive to more long-lasting plasmolysis is lethal to *N. meningitidis* cells, and that membrane damage plays a part in this lethality.

N. meningitidis cells that had been exposed to hypotonic conditions were also drastically changed with regard to osmotic properties. There is evidence for a change in permeability for a number of solutes. Unlike the situation in intact cells, several solutes such as phosphate can no longer establish an osmotic gradient at all. These findings may indicate that plasmoptysis occurs in *N. meningitidis* and that the phenomenon is followed by membrane damage.

As observed in other bacteria (20) cells in the exponential phase were generally more sensitive than stationary phase cells. This may partly be connected with cell division. The first, rapid phase of killing during exposure to distilled water which is particularly evident in cp^+ cells (Fig. 4) may represent that part of the cell population in which a part ("ribbon") of the cell wall is weakened during a particular phase of the division cycle (20). It may be of interest that a difference between the regulation of cell division in cp^+ and cp cells has previously been noted (14 15).

This work suggests strongly that the degree of membrane damage is constantly less in the cp variant than in the cp^- one. One may speculate how the surface structure could convey upon the cp^+ cell a pronounced capacity for shrinkage or swelling. One possibility is that both competence variants are provided with a rigid cell wall to which the membrane is quite firmly attached, but that the cp cell in addition is equipped with membrane invaginations or a true mesosome that can be "unfolded" upon removal of intracellular water. This would provide the cp^+ cell with an extra protection against harmful effects of shrinkage such as plasmolysis. The study of *Bideau* (3) indeed indicates that *N. meningitidis* may have structures equivalent to mesosomes. Several workers have observed that mesosomes extrude or evaginate during the formation of protoplasts, or with comparatively slight osmotic change (7). The presence of mesosome-like structures might also explain the relative resistance of cp cells when exposed to low tonicity although it might also be that the cell wall of the cp^+ cell is different, with a higher tensile strength.

The author is greatly indebted to Miss *Lydia Gihle* for excellent technical assistance.

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5161 CIP 6444 and CIP 6447 were also kindly supplied by Dr *P Thibault* and were isolated by Dr *R. Vincent* from cases of gingivitis (CIP 5161) pyorrhea (CIP 6444) and appendicitis (CIP 6447) (personal communication from Dr *R. Chatelain Institut Pasteur Paris France*)

T microdentium strain FM was kindly supplied by Professor *P H Hardy* Department of Microbiology Johns Hopkins University Baltimore, USA, who originally obtained the strain from Professor *E. G Hampp*

The treponemes were grown in a thioglycollate medium, as described previously (3) and cells were harvested by centrifugation as stated earlier (2)

All treatments with the detergents, Teepol and sodium deoxycholate, and with the enzyme *Myxobacter* AL-1 protease I (AL-1 enzyme) as well as *preparations for negative staining and procedures* for electron microscopy were carried out as described in detail in a previous paper (2)

RESULTS

Treponema calligyrum

Organisms of *T calligyrum* were found to be regularly coiled (Fig 1) with a mean wavelength of 1 15 μm and an amplitude of about 0.3 μm. Their length, as measured along the axis of the helices, varied between 8 and 12 μm and the width was 0 16 to 0 19 μm. The cells were covered by a rather loose, regularly structured surface layer (Fig 4) The ends of the organisms were tapered with two or three flagella inserted at each end (Fig 4) Cells with one flagellum inserted at one or both ends were seen only twice. The most proximal flagellum was inserted about 0 1 μm from the end of the cytoplasmic body of the cell. The bundles of flagella, one bundle originating from each end, were twined together with the cytoplasmic body of the cell and the flagella overlapped in the middle of the organism where they interdigitated.

Treatment with 0 2 per cent Teepol for 2 minutes straightened the treponemes somewhat, and their cell bodies showed a mottled appearance (Fig 7) The surface layer seemed to be loosened by this treatment and was found as isolated flakes on the supporting membrane of the grid (Fig 7) Some thin fibrils with a diameter of about 2 nm could be observed in between the flakes (Fig 7) These fibrils appeared to originate from slightly damaged regions of the regularly structured surface layer Only a few flagella were freed from the cells by treatment with Teepol (Fig 7)

Treatment with 1 per cent sodium deoxycholate for 2 minutes had little effect on the shape of the treponemes or the insertion of their flagella. The regularly structured surface layer still covered the cells (Fig. 10). Some micrographs showed thin fibrils with a diameter of about 2 nm protruding from the layer Sodium deoxycholate treatment did, however have some effect on the treponemes as cytoplasmic tubules or microtubules with a diameter of 7 nm (2) were observed in the interior of cells after this treatment (Fig. 10)

The effect of AL-1 enzyme on *T calligyrum* cells varied from cell to cell. Only flagella, bundles of cytoplasmic tubules and membranous debris remained of some of the cells treated either for 30 seconds or 2 minutes, while other cells appeared unaffected after treatment for 2 minutes. All treponemes were damaged after exposure to AL-1 enzyme for 4 minutes (Fig 11)

Two bundles of cytoplasmic tubules were observed in the treponemes after treatment with AL-1 enzyme. A bundle seemed to originate from each end of the cell and consisted of 6 to 8 individual tubules with a diameter of about 7 nm (Figs. 11 & 12) *As many as* 12–14 microtubules were present in the middle of the organism (Fig 12)

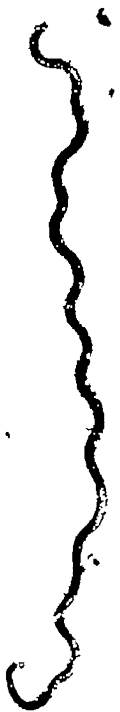
All figures show material negatively stained with 1 per cent ammonium molybdate. The bar on each micrograph represents 100 nm unless otherwise stated.

Figs. 1–6 all show cells from unfixed preparations.

Fig 1 T calligyrum cell with regular waves and somewhat tapered ends. × 15,000.

Fig 2 T minutum Note the tapered ends of this regularly coiled treponeme. × 15,000.

Fig 3 T microdentium (strain FM) Regularly waved organism with blunt ends. × 22,500.







1µm 2 1µm 3 1µm

The flagellum consisted of a shaft, a hook and a basal knob (Fig 13) Generally the *shaft of the flagellum was covered by a* sheath, but sometimes this sheath was accidently removed during the preparation procedure. Such a "naked" shaft or core had a diameter of about 11 nm, while the diameter of the sheathed flagellum was *16–19* nm. The hook which was approximately 50 nm long and 15 nm wide, showed a characteristic *honeycombed substructure (Fig 13) A col*lar 16–18 nm long and about 9 nm wide connected the hook to the basal knob (Fig 13) The insertion regions of the flagella were usually obscured by adhering membranous debris. *In cells treated with AL-1* enzyme the flagellar insertion region appeared as electron dense plates with a diameter of about 25 nm surrounded by a less electron dense ring about 14 nm wide (Fig 11) An indication of a substructure in this ring could be discerned on some micrographs.

Isolated flakes of the regularly structured surface layer *were found in preparations of* flagella isolated by differential centrifugation (Fig 13) The substructural pattern of this layer seemed to be produced by tightly packed rows of small rings or polygons *with a centre-to-centre distance of 10 nm.*

Treponema minutum

Cells of *T minutum* were regularly coiled (Fig. 2) The length of individual organisms varied from 9 to 18 μm and the width was 0 17 to 0 19 μm. The mean wavelength was 1.36 μm and the amplitude *0.20* μm. *The cells were covered by a regularly structured* surface layer (Fig 5) The ends of the treponemes were tapered with two to three flagella inserted at each end (Fig 5) The most proximal flagellum was inserted about 0 1 μm *from the end of the cytoplasmic body* of the cell. The two bundles of flagella, one originating from each end, were twined together with the cell body and the flagella overlapped and interdigitated in the mid-region.

The *regular* substructure of the surface layer was destroyed when the treponemes had been treated with Teepol for 30 seconds (Fig 8) This effect of Teepol on the regular structure of the surface layer was more pro*nounced* when the treatment was prolonged to 2 minutes, after which the regular structure was barely discernible among thin fibres with a diameter of 4–5 *nm* (Fig. 14) Almost all the flagella were freed from organisms treated with Teepol for 2 minutes (Fig. 14).

After treatment with sodium deoxycholate for 2 minutes the regularly structured sur*face layer was still recognizable and* cytoplasmic tubules were visible in the cytoplasmic body of the treponemes (Fig. 15)

The treponemes retained their wavy outline after treatment with Teepol as well as with sodium deoxycholate.

The effect of AL-1 *enzyme* on *T minutum* cells varied from organism to organism in the same manner as described above for *T calligyrum* cells. Bundles of cytoplasmic tubules

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Fig 4 T calligyrum. The substructure in the surface layer of the cell (arrow) is clearly seen. Note the tapered end with two flagella inserted (I). × 90,000.

Fig 5 T minutum A tapered end of a cell is shown. The cell is covered by a regularly structured surface layer (arrow) The insertions (I) of two flagella are also seen. × 90,000.

Fig 6 T microdentium (strain FM) The cell is covered by a *regularly* structured surface layer (arrow) Note the blunt end with two flagella (F) *inserted (I)* × 90,000.

Figs 7–9 show cells treated with 0 2 per cent Teepol for 30 seconds (*Figs 8 & 9*) or 2 minutes (Fig. 7)

Fig 7 T calligyrum. The flagella (F) are still connected to the organism. Thin fibrils (arrows) are seen in the ruptured or damaged parts of the surface layer (SL) *Note the mottled appearance of* the cytoplasm. × 90,000.

Fig 8 T minutum. The regularly structured surface layer (SL) is somewhat destroyed and thin fibrils are visible (arrow) The flagella *(F)* are inserted in the cytoplasm of the cell. × 90,000.

Fig 9 T microdentium (strain FM) The two flagella (F) are torn *out* of the cell cytoplasm The substructure of the surface layer is not recognizable after the Teepol treatment. × 90,000


4
I


5
I


6
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I
F

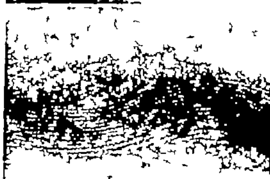

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SL
F

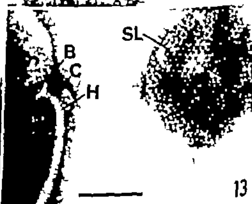

8
SL
F


9
F









Figs 10–13 all show material obtained from cultures of *T calligyrum*.

Fig 10 Treatment with sodium deoxycholate for 2 minutes has revealed cytoplasmic tubules (T) in the interior of the cell. The substructure of the surface layer is only slightly affected by the treatment. × 90,000.

Fig 11 Part of a treponeme treated with AL-1 enzyme for 4 minutes. Flagella (F) and cytoplasmic tubules (T) are recognizable together with some flakes of the surface layer (SL). The ends of the cytoplasmic tubules are seen close to the insertion region of a flagellum (arrow). Note the ring-shaped structure (R) surrounding the basal end of the flagellum. × 160,000.

Fig 12 The two bundles of cytoplasmic tubules are shown to overlap in the middle part of a cell treated with AL-1 enzyme for 2 minutes. × 90,000.

Fig 13 A flagellum liberated after treatment of suspended cells with Teepol. The basal knob (B) the collar (C) and the book (H) are seen. The substructural pattern of rows of tightly packed rings or polygons is illustrated on the flake of the surface layer (SL) present in the upper right part of the field. × 160,000.

TABLE 1 *Dimensions of Cells and Flagella of* T calligyrum, T minutum and T microdentium

Species	*T calligyrum*	*T minutum*	*T microdentium*			
Strain	CIP 6441	CIP 5162	FM	CIP 5161	CIP 6444	CIP 6447
Length*	8–12 μm	9–18 μm	4–8 μm	7–10 μm	6–10 μm	6–9 μm
Diameter*	0.16–0 19 μm	0 18 μm	0.18 μm	0.15 μm	0 17 μm	0.17 μm
Ends	Tapered	Tapered	Blunt	Blunt	Blunt	Blunt
Number of flagella	2–3 (2.5)	2–3 (2.5)	1–3 (2.1)	1–3 (2.3)	1–3 (1.9)	1–3 (2.0)
Wavelength	1 15 μm	1.36 μm	0.9 μm	0.9 μm	0.9 μm	0.9 μm
Amplitude	0.3 μm	0.2 μm	0 14 μm	0.14 μm	0.15 μm	0.16 μm
Distance from tip to outer most inserted flagellum	0.1 μm	0.1 μm	0.1 μm	0 1 μm	0.1 μm	0.1 μm
Diameter of sheathed flagellum	18 nm	18 nm	19 nm	18 nm	18 nm	18 nm
Diameter of naked flagellum	11 nm	11 nm	12 nm	11 nm	12 nm	11 nm
Diameter of hook	15 nm	14 nm	15 nm	15 nm	15 nm	15 nm
Length of hook	50 nm	50 nm	50 nm	50 nm	50 nm	50 nm
Diameter of collar	9 nm	9 nm	10 nm	9 nm	9 nm	–
Length of collar	16 nm	15 nm	15 nm	15 nm	15 nm	–
Diameter of basal knob	35 nm	30–35 nm	35 nm	30–35 nm	35 nm	–

The range is given for the dimensions marked with asterisks; all other figures are mean values.
– not measured.

were visible in cells affected by the treatment. Each bundle consisted of 6–8 tubules with a diameter of 7 nm (Fig. 16)

Flagella isolated from *T minutum* were in all respects identical to flagella isolated from *T calligyrum* (Table 1 Figs. 17 & 18)

Isolated flakes of the regularly structured surface layer were sometimes observed close to unfixed treponemes which were accidently damaged during preparation. The substructure appeared to be well preserved as compared to the substructure on undamaged organisms, but the pattern was difficult to resolve. It seemed to consist of rows of round or polygonal depressions in which the heavy metal salt used for negative staining had accumulated (Figs. 19 & 20) The interstices between each of the depressions presented a somewhat uneven texture, as of a fabric woven with delicate threads. The centre-to-centre distance between each depression was 8–10 nm (Figs. 19 & 20) On a few micrographs some pieces of isolated surface layers presented serrated edges (Fig. 19)

Treponema microdentium Strain FM

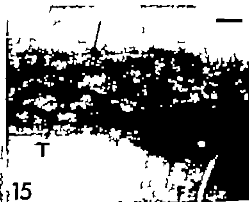
The length of the cells of this regularly coiled treponeme varied between 4 and 8 μm as measured along the axis of the helices, and the width was about 0.18 μm (Fig 3) The mean wavelength was 0.9 μm and the amplitude 0.13–0.14 μm. The cells were found to be covered by a regularly structured surface layer (Fig. 6) The ends of the cells were blunt, and two or three flagella were inserted at each end (Fig. 6) 18 out of 30 treponemes examined had two flagella and 6 had three flagella inserted at each of the two ends of the cells. The remaining 6 had two flagella inserted at one end and three (or one) at the other. The most proximal flagellum was inserted about 0 1 μm from the end of the cytoplasmic body of the organism. The flagella were twined together with the cytoplasmic body of the treponeme and interdigitated in the middle of the cell.

Almost all the flagella were freed from the treponemes by treatment with 0.2 per cent Teepol for 30 seconds (Fig. 9)

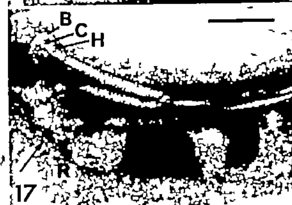
Bundles of cytoplasmic tubules were revealed in the cytoplasm of organisms treated with sodium deoxycholate (Fig 21)

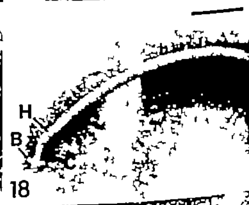
The effect of AL-1 enzyme varied from cell to cell, but generally the cells became more damaged after prolonged treatment with the enzyme. Only flagella, bundles of


14
F
F

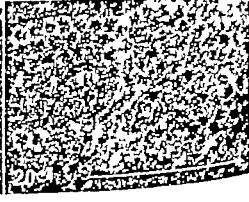

T
F
15


T
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16


B
C
H
R
17


H
B
C
18


19


20

cytoplasmic tubules and membranous debris were left of the most affected organisms (Fig 22) Each bundle of cytoplasmic tubules consisted of 5–6 tubules, with a diameter of about 7 nm for each individual tubule (Fig. 22)

Flagella isolated from *T microdentium* strain FM were in all respects identical to flagella isolated from *T calligyrum* and *T minutum* (Table 1 Fig 23)

The regular substructure of the surface layer could be resolved into rows of tightly packed round or polygonal depressions with a centre to-centre distance of about 10 nm (Fig. 24) This regular substructure was destroyed on cells treated with Teepol or sodium deoxycholate. Thin fibrils with a diameter of 1.2 to 2 nm were visible close to cells treated with the detergents.

Figs 14–20 all show material obtained from cultures of *T minutum*.

Fig 14 The flagella (F) are liberated from this cell which was treated with Teepol for 2 minutes. Thin fibrils (arrow) are visible in the remains of the surface layer Note the mottled appearance of the cytoplasm. × 160,000.

Fig 15 Cytoplasmic tubules (T) are seen in a cell which was treated with sodium deoxycholate for 2 minutes. Thin fibrils (arrows) are visible in association with the regularly structured surface layer F denotes flagellum. × 90,000.

Fig 16 Remnants of a cell which was treated with AL-1 enzyme for 4 minutes. The bundle of cytoplasmic tubules (T) consists of 6 individual tubules. Note the substructure (arrow) of the surface layer F denotes flagella. × 90,000.

Figs 17 & 18 Flagella liberated from cells which were treated with Teepol for 1 minute. Basal knobs (B) collars (C) and hooks (H) are seen. Arrow (Fig. 17) points to remnants of the surface layer and R denotes ring-shaped structure surrounding the basal end of the flagellum. × 160,000.

Figs 19 & 20 Isolated flakes of the surface layer of an unfixed cell accidently damaged during preparation for electron microscopy Note the serrated edge in Fig. 19 and the rows of round or polygonal depressions in Fig. 20. Arrows point to regions where the woven texture of the interstices is well resolved. × 410,000.

Treponema microdentium, Strains CIP 5161 CIP 6444 and CIP 6447

The length of the organisms of these strains varied from 6 to 10 μm and was consequently slightly longer than that of cells of *T microdentium* strain FM. Apart from this variation, all gross morphological and ultrastructural characteristics of cells from the four different strains of *T microdentium* studied were identical or of the same order of magnitude (Table 1)

Statistical Analysis

For the statistical analysis it was presumed that the observed measurements of cell lengths and wavelengths could be described as mutually independent, normally distributed stochastic variables with parameters dependent on the strain in which the measurements were performed.

The assumption of normality was confirmed, since histograms of the cell lengths and wavelengths showed no systematic deviation from normal distributions. The histograms are not shown in this paper

One-way analysis of variance was used to test whether the variances and the means could be assumed to be identical within strains of the same species and within the different species.

The results for the four strains of the species *T microdentium* are shown in Table 2. The distribution of the cell lengths did not show the same variance in the four strains, and furthermore the mean value in strain FM was considerably lower than in the other three. The distribution of the wavelengths was found to be identical for all four strains (test level 50 per cent)

When a test was carried out to ascertain whether the mean cell lengths and the mean wavelengths of the three species were different, the values for *T microdentium* were treated as if they were derived from one strain.

From Table 3 it can be seen that the variances between cell lengths were not identical, since the species with the longest cells (*T*



Figs 21–24 all show material obtained from cultures of *T microdentium* strain FM

Fig 21 Cytoplasmic tubules (T) are visible in this cell which was treated with sodium deoxycholate for 2 minutes. The surface layer (SL) covers the cell, and the flagella (F) are inserted in the cytoplasm. × 90 000.

Fig 22 Cell after treatment with AL-1 enzyme for 30 seconds. Flagella (F) cytoplasmic tubules (T) and membranous debris (D) are seen. Flakes of the surface layer in which the substructure is recognizable (arrows) are also present. Note the ring (R) surrounding the basal end of one of the flagella × 90,000.

Fig 23 An isolated flagellum obtained after treatment of cells with Teepol for 2 minutes. Basal knob (B) collar (C) and hook (H) are seen. × 160,000.

Fig 24 Flakes of the regularly structured surface layer obtained after treatment of a cell suspension with AL 1 enzyme. × 180,000

TABLE 2. *The Means and Variances of Cell Lengths and Wavelengths for Four Strains of* Treponema microdentium

Strain	No. of observations	Cell lengths			Wavelengths		
		$\bar{x}$	s^2	SE	$\bar{x}$	s^2	SE
6444	7	7.71	2.28	0.57	0.99	0.012	0.04
6447	7	7.37	0.21	0.17	0.94	0.039	0.07
5161	7	8.16	1.35	0.44	0.91	0.007	0.03
FM	12	6.14	1.46	0.35	0.91	0.006	0.02
Common variance						0.014	
Total	33	7.16	1.90	0.24	0.93	0.014	0.02

$\bar{x}$ = mean.
s^2 = variance.
SE = standard error of the mean.

TABLE 3. *The Means and Variances of Cell Lengths and Wavelengths for* T. calligyrum, T. minutum *and* T. microdentium

Species	No. of observations	Cell lengths			Wavelengths		
		$\bar{x}$	s^2	SE	$\bar{x}$	s^2	SE
T. calligyrum	8	10.68	1.53	0.44	1.13	0.013	0.04
T. minutum	11	13.08	9.78	0.94	1.36	0.029	0.05
T. microdentium	33	7.16	1.90	0.24	0.93	0.014	0.02

$\bar{x}$ = mean.
s^2 = variance.
SE = standard error of the mean.

minutum) showed the greatest variance. It is of special interest to note that the result for *T. microdentium* derived from four different strains did not show the largest variation. The mean cell lengths were obviously different for all three species studied (Table 3).

The variances of the wavelengths were the same for all the species (Table 3). The three species had mutually different mean wavelengths (test level 0.5 per cent) (Table 3).

DISCUSSION

The results of the measurements performed on cells of the strains investigated and the flagella isolated from them are listed in Table 1.

Plots of corresponding pairs of lengths and wavelengths for cells of the two species, *T. microdentium* and *T. minutum* showed no mutual dependence. For *T. calligyrum* however there was a tendency of an increasing wavelengths with increasing cell length.

Due to the demonstration of independence of length and wavelength of the cells of the first two species mentioned, it seemed justifiable to perform parallel tests for the two kinds of observations (the diagrams are not shown in this paper).

The cell lengths varied both with respect to variances and to mean values within strains of the same species and particularly between the three species.

The wavelengths of the strains of all three species were assumed to have common variance when further analyses were carried out. The results of these analyses were that the four strains of *T. microdentium* could be

considered to have a common mean while the three species had different means.

In addition to the difference in mean cell length and wavelength, the cells of *T microdentium* also have a smaller amplitude than those of *T minutum* and *T calligyrum*

The effect of treatment with Teepol on cells of the three species examined varied from species to species. This variation may be of use for the identification of organisms of these species, and may be briefly summarized as follows

1 The substructure of the regularly structured surface layer of *T minutum* and *T microdentium* was heavily disorganized after the treponemes had been treated with 0.2 per cent Teepol for 30 seconds, whereas the substructure of this layer on *T calligyrum* cells seemed to be unaffected even after 1½ hours of treatment.

2. Almost all the flagella were liberated from *T microdentium* after 30 seconds of treatment with 0.2 per cent Teepol, whereas this treatment only liberated a few flagella from *T minutum* and *T calligyrum* When the treatment was prolonged to 2 minutes, most of the flagella were liberated from *T minutum* while they remained attached to *T calligyrum*.

Individual organisms showed great variation in the ability to resist treatment with AL-1 enzyme. This variation was of the same order of magnitude for all the strains studied and might be due to individual cells having different degrees of resistance to the effects of the enzyme, depending on the age of the cells or other physiological factors.

Micrographs in which the regular substructure of the surface layer of the treponemes is clearly depicted leave the impression that the pattern is different for each species (Figs. 18 20 24) but that it is very similar if not identical, for all the four strains of *T microdentium* studied. Optical diffraction analysis is currently being performed to test this hypothesis.

All characteristics of the investigated strains of *T microdentium* are very similar Consequently the classification of the four strains of *T microdentium* (FM, CIP 5161, CIP 6444 and CIP 6447) within the same species, seems justifiable from a morphological point of view Cells of *T microdentium* differ clearly from those of *T minutum* and *T calligyrum* with respect to amplitude, wavelength and mean cell length. Furthermore the cell ends of *T microdentium* are blunt, whereas those of *T minutum* and *T calligyrum* are tapered. Cells of *T minutum* and *T calligyrum* differ from each other by having different amplitudes, wavelengths and cell length distributions, as well as by the different patterns presented by their regularly structured surface layer

Consequently the conclusion of the present work is that the morphological differences between cells of *T microdentium*, *T minutum* and *T calligyrum* justify maintaining these as separate species. Previously *[illegible] & Gruntes* (6) concluded that the four strains they studied *viz.* *T calligyrum*, *T minutum* *T refringens* and *T phagedenis*, were characterized by differences in structure, and the strains of *T calligyrum* and *T minutum* they used were the same as those studied in the present work.

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PURINE METABOLISM IN *NEISSERIA MENINGITIDIS*

I. *Utilization of Exogenous Adenine*

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Whole cells of *Neisseria meningitidis* are found to utilize exogenous radioactive adenine with labelling of the adenosine 5'-monophosphate, adenosine 5'-diphosphate and adenosine 5'-triphosphate pools. Radioactive hypoxanthine or adenosine were not found in ethanolic extracts from whole cells. Crude extracts from *N. meningitidis* were found to have activities corresponding to adenine phosphoribosyltransferase (EC 2.4.2.7). The adenosine 5'-monophosphate formed was phosphorylated to adenosine 5'-triphosphate. No activity corresponding to purine-nucleoside phosphorylase (EC 2.4.2.1) or to adenine deaminase (EC 3.5.4.2) could be demonstrated in crude extracts from *N. meningitidis*.

In an earlier report it has been shown that *Neisseria meningitidis* lacks the enzymes known to be involved in the utilization of exogenous thymine or thymidine for incorporation into DNA (17). Attempts to isolate spontaneous or mutagen-induced mutants requiring thymine or thymidine were altogether negative. Selective procedures involving folic acid analogues gave only one class of mutants with requirements involving precursors of nucleic acids. This class comprised mutants which required adenine plus guanine for growth (19).

In the present work the growth of an adenine-guanine mutant in the presence of purines and purine intermediates has been examined. The growth of the corresponding prototrophic strain M1 of *N. meningitidis* in the presence of the base analogues 2,6-diaminopurine and 6-mercaptopurine has also been measured, and the labelling of purine intermediates from radioactive adenine taken up by whole cells has been examined. Subsequently enzyme functions known to be involved in the utilization of adenine have been measured. Some properties of the enzyme adenine phosphoribosyltransferase have been studied.

Three known ways exist by which adenine may be utilized for conversion into its corresponding 5'-ribomononucleotide, either directly or indirectly (Fig. 1).

I. Adenine phosphoribosyltransferase, EC 2.4.2.7 (4): adenine + 5-phospho-α-D-ribose 1-diphosphate (*PRPP*) ⇌ adenosine 5'-monophosphate + pyrophosphate (PP_i)

II. Purine-nucleoside phosphorylase, EC 2.4.2.1 (4): purine nucleoside + orthophosphate ⇌ purine + α-D-ribose 1-phosphate (R-1-P)

This last reaction is readily reversible (32). For conversion of the adenosine formed, to AMP an active adenosine kinase has to be present, or the adenosine may be deaminated to inosine which in turn can be converted to

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trifugation at 4 C the cell residue was re-extracted in an identical manner with 0.5 ml 50 per cent ethanol. The combined extract was treated as before and examined by chromatography (17).

Separation, identification and estimation of radioactive metabolites. The procedures were essentially as before (17). In Table 1 are listed the standards which were used for identification of radioactive metabolites in ethanolic extracts of whole cells after exposure to ^{14}C purine bases and ribonucleosides. The chromatographic behaviour of the standards is given in terms of $R_{adenine}$, the distance moved by the standard relative to that moved by adenine. $R_{adenine}$ was not used for identification in the actual experiment. The labelled spots were compared with authentic, unlabelled samples of the suspected compounds by observation in ultraviolet light after chromatography and radioautography (17). The separation of neighbouring spots varied from chromatogram to chromatogram. The chromatograms with the radioactive spots were cut in squares, and these were counted in the Packard Tri-Carb Spectrometer (17). The counting efficiency of the carbon 14 compounds was 54 per cent.

Adenine phosphoribosyltransferase EC 2.4.2.7 (4). Unless otherwise noted this enzyme activity was assayed in a total volume of 100 µl by mixing 100 µmoles potassium phosphate buffer pH 7.4, 0.1 µmole $MnCl_2$, 0.05 µmole PRPP, 0.0083 µmole ^{14}C-8-adenine (60 µCi/µmole) and 25 µl crude extract. The reaction was initiated by the addition of extract and carried out at 37 C in a shaking water bath. The reaction was run for 30 minutes, or as indicated in the individual experiments, and stopped by cooling in an ice-water bath. The protein was precipitated with 100 µl ice-cold 96 per cent ethanol followed by 10 µl 0.1 M potassium EDTA and removed by centrifugation. Appropriate controls with deproteinized extracts were included. Separation of adenine from adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP) and adenosine 5'-triphosphate (ATP) was achieved according to *Hochstadt-Ozer & Stadtman* (9). Cellulose thin layers 20 × 20 cm (Eastman Chromagram with fluorescent indicator) were ruled in channels, 1.5 × 8 cm, and the samples, 5 or 10 µl of the supernatants, were applied between the pencilled lines. The thin layer origins were spotted with unlabelled adenine and AMP 5 µg of each, to facilitate detection of spots under ultraviolet light after development of the chromatograms. The chromatograms were developed by ascending chromatography in the Eastman Chromagram developing apparatus in 1 M NH_4-acetate pH 7.0 in about 10 minutes (6 cm). Adenine, AMP, ADP and ATP had R_F values of 0.31, 0.67, 0.74 and 0.80 respectively. The spots were marked with a pencil and strips from the position of AMP to the solvent front were cut and counted as mentioned. The adenine phosphoribosyltransferase activity is given as the sum of the ribonucleotides formed AMP + ADP + ATP. No radioactivity at the R_F 0.51 and 0.55, adenosine and hypoxanthine respectively, was detected, indicating the absence of interfering enzyme activities leading to the synthesis of these metabolites in crude meningococcal extracts. This was confirmed by chromatography for 3 hours (17 cm) in the n-butanol solvent (Table 1), which also showed the lower spot to be unlabelled. This solvent separates AMP, ADP and ATP (R_F 0.32, 0.17, 0.11) from adenine, adenosine, hypoxanthine and inosine (R_F [illegible], 0.65, 0.59, 0.55).

Purine-nucleoside phosphorylase EC 2.4.2.1 (4) was assayed according to *Hochstadt-Ozer & Stadtman* (10) by mixing in a total volume of 50 µl 5 µmoles Tris/HCl buffer pH 7.8; 1.5 µmoles $MgCl_2$, 0.15 µmole R-1-P, 0.05 µmole ^{14}C-8-adenine (10 µCi/µmole, adjusted to this activity with the nonradioactive compound prior to use) and 10 µl crude extract. The experiments were run for 5 minutes (*E. coli*) and 30 minutes (*N. meningitidis*). Precipitation of protein was with 50 µl 96 per cent ethanol and 5 µl 0.1 M EDTA. The rest of the procedure was as described for adenine phosphoribosyltransferase, except that the chromatography in 1 M NH_4-acetate was for 45 minutes (17 cm) and with 2 cm between the origins. The unlabelled metabolites studied were spotted as carriers. The extracts in these experiments were made from exponentially growing cultures (see Media) with approximately 1.5 × 10^8 C.f.u./ml. Aliquots of the cultures were diluted with an equal volume of fresh prewarmed medium containing 2 mM adenosine final concentration for possible enzyme induction (6). Growth was continued for 90 minutes. Cells were harvested by centrifugation, washed and suspended in 0.1 M Tris/HCl pH 7.6 with 10 mM dithiothreitol (DTT) (approx. 0.1 g wet cells in 1 ml buffer). Sonication and centrifugation were as before (17). The supernatant was dialysed overnight against 100 volumes 0.05 M Tris/HCl pH 7.6 plus 2 mM DTT. The *E. coli* extracts were diluted 1 to 10 with Tris/HCl buffer 0.1 M pH 7.6—10 mM DTT before use.

Adenine deaminase EC 3.5.4.2 (4) was assayed according to *Lawrence* (26). In a total volume of 1.5 ml 150 µmoles Tris/HCl buffer pH 7.4 were mixed with 5 or 0.2 µmoles adenine and 0.5 ml crude extract with 2.7 mg protein. The reactions were run for 15, 30, 60, 90 and 120 minutes at 37 C with shaking and terminated with addition of 1.5 ml 4 per cent perchloric acid. After centrifugation, 0.1 ml of the supernatant if 5 µmoles adenine were used, and 1 ml of the supernatant if 0.2 µmole adenine was used, was mixed with water to 2.5 ml. The spectra were then recorded in a Hilger-Gilford spectrophotometer from 240 to 290 nm. Adenine and its derivatives have absorption

peaks at 260 nm while the peak absorption of hypoxanthine and its derivatives are at 250 nm (20, 21)

Adenylate kinase EC 2.7.4.3 (4) A reaction volume of 100 µl consisted of 10 µmoles of Tris/HCl buffer pH 7.6 1 µmole $MgCl_2$ 0.5 µmole ATP 0.0046 µmole ^{14}C-8-AMP (54 µCi/µmole) and 25 µl crude extract. The reactions were run for 5, 10 15 30 and 60 minutes. The rest of the procedure was similar to that used in the thymidine kinase assay with thin layer chromatography in one dimension in the isobutyric acid solvent (16) The R_F of the metabolites are AMP 0.67 ADP 0.57 and ATP 0.49

Chemicals. ^{14}C-8-adenine (60 mCi/mmole) and ^{14}C-8-AMP ammonium salt (54 mCi/mmole) were obtained from The Radiochemical Centre, Amersham, Bucks. U.K. 5-aminoimidazole-4-carboxamide ribonucleotide (AICA-R) was obtained from Boehringer Mannheim, GmbH (Mannheim, Germany) Other fine chemicals were from Koch-Light Labs. Ltd. Bucks. U.K. or from Sigma Chemical Corporation, St. Louis, Mo. U.S.A. ^{14}C-labelled and unlabelled purine bases, nucleosides and ribonucleotides were checked for purity by thin layer or paper chromatography observation in ultraviolet light and when radioactive, with radioautography The 5-phospho-α-D-ribose 1-diphosphate tetrasodium salt (PRPP) was from Sigma. It was stored at −100° C, and solutions were kept at 0° C prior to use. The actual concentration of PRPP in solution was determined by its capacity to convert ^{14}C-adenine to ^{14}C-AMP in the reaction catalysed by *E. coli* adenine phosphoribosyltransferase. The reaction goes to completion (9 34) and limiting amounts of PRPP were reacted with excess of adenine and enzyme. Crude *E. coli* extract dialysed against 0.05 M Tris/HCl buffer pH 7.8 overnight was used The reaction mixture contained in a total of 50 µl 4 µmoles Tris/HCl buffer pH 7.8 0.2 µmole $MgCl_2$ 0.05 µmole ^{14}C-8-adenine (10 µCi/µmole) 0.005 µmole PRPP by weight, and

TABLE 2 *Growth of the Mutant 6-1b of* N. meningitidis *on Different Purine Sources*

Adenine	—	Adenine + xanthosine	—
Guanine	—	Adenosine + guanine	+
Hypoxanthine	+	Inosine	—
Xanthine	—	Adenine + GMP	—
Adenine + guanine	+	Guanine + AMP	—
Adenine + xanthine	+	IMP	—
Adenine + guanosine	—		

The mutant was grown on Medium A plates supplemented with histidine proline and the above mentioned purine sources (27) Incubation was in an air tight jar with humid atmosphere with approximately 5 per cent CO_2 at 37 C (15)

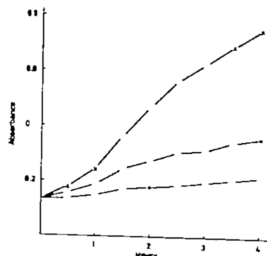


Fig 2 Inhibitory effect of purine analogues (conc. 2.1 × 10^{-3} M) on the growth of *N meningitidis* in KC medium. Growth at 37 C with shaking was followed in a Beckman C colorimeter (17) No addition (×—×) 2,6-diaminopurine (○—○) and 6-mercaptopurine (●—●) added.

10 µl crude extract with about 0.1 mg protein. The reaction was run for 10 15 and 20 minutes. The rest of the procedure was as for the *N meningitidis* adenine phosphoribosyltransferase with chromatography in the n-butanol solvent (Table 1) The crude dialysed extracts from *E. coli* convert some of the AMP formed to ADP and ATP The PRPP was found to be 50 per cent pure. This is in accordance with the assay of the producers.

RESULTS

Growth Studies

Nutritional requirements The purine mutant 6-1b of *N meningitidis* requiring the amino acids histidine, proline and the purine bases adenine and guanine for growth was tested for its ability to utilize other purine compounds as shown in Table 2. The mutant is unable to utilize exogenous adenine, guanine or xanthine as the sole purine source, but grows well on hypoxanthine. Among the purine precursors examined, only xanthine could substitute for guanine, whereas adenosine could substitute for adenine.

Growth in the presence of base analogues The effect of the base analogue 2 aminopurine on the growth of *N meningitidis* strain M1 has been examined before. In

TABLE 3. *Labelling of Purine Metabolites from ^{14}C-8-adenine in Intact Cells*

Organism	Time of incubation sec	Incorporation calculated as picomoles adenine					
		A	Hx	AR	I	AMP + ADP + ATP	GMP + GDP + GTP
N meningitidis							
Strain M1	15	20.8	–	–	–	124.6	3.2
	60	10.2	–	–	–	191.5	6.7
	120	5.0	–	–	–	234.0	8.0
E. coli K12	15	54.0	30.3	23.3	4.4	613.9	73.4

The assay was as described in Methods.

Medium A the growth is only 82 per cent with 2 aminopurine in a concentration of 1×10^{-4} M (19) Fig. 2 shows the inhibition of growth of strain M1 in Medium KC devoid of adenine and guanine with 2,6-diaminopurine and 6-mercaptopurine, both in the concentration of 2.1×10^{-4} M. There is a pronounced effect on the growth of *N meningitidis* with both analogues. This points to active adenine and guanine-hypoxanthine phosphoribosyltransferases in *N meningitidis* (22, 25)

Labelling of purine metabolites from ^{14}C-8-adenine The labelling of AMP ADP and ATP from ^{14}C-8-adenine added to cells of *N meningitidis* strain M6 has been demonstrated earlier (17) A re-examination of the labelling from ^{14}C-8-adenine was performed with *N meningitidis* strain M1 *E. coli* was simultaneously examined to provide a means for comparison with better known systems (11) Table 3 shows that adenine also in strain M1 of *N meningitidis* is incorporated into AMP ADP and ATP whereas no label from adenine appeared in adenosine, hypoxanthine or inosine. A faint labelling of the guanosine 5'-nucleotides GMP GDP and GTP also occurred. In *E. coli* adenine is rapidly incorporated into the adenosine 5'-nucleotides and guanosine 5'-nucleotides. The spots corresponding to adenosine, hypoxanthine and inosine also showed radioactivity (Table 3)

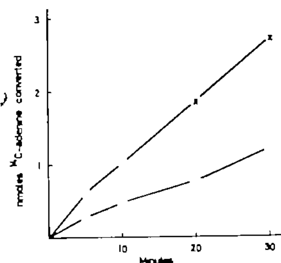


Fig 3 Activities corresponding to adenine phosphoribosyltransferase in crude meningococcal extract. Experiment A (×—×) was performed with 25 µl extract containing 0.29 mg protein. In experiment B (●—●) half as much extract was used as in experiment A. Experimental conditions as described in Methods.

Adenine Phosphoribosyltransferase

Effect of enzyme concentration and time Fig 3 shows the enzyme activity related to time. In experiment B, half as much extract was used as in experiment A. Each point represents the sum of the adenosine 5'-nucleotides formed (AMP + ADP + ATP) because of an active AMP and ADP kinase present in the crude extracts. The kinases require phosphate donors and divalent cations for activity Phosphate donors are obviously present in crude extracts, and both Mn^{++} and Mg^{++} may be used as cations in the reactions, as found in other systems (31 33)

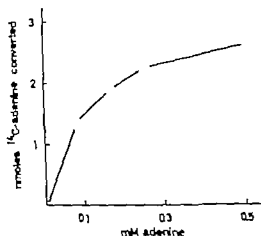


Fig 4 Activities corresponding to adenine phosphoribosyltransferase in crude meningococcal extract. Effect of adenine concentration. The assay was performed as described in Methods with 25 µl extract containing 0.22 mg protein.

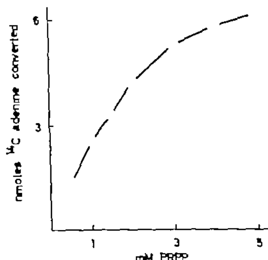


Fig 5 Activities corresponding to adenine phosphoribosyltransferase in crude meningococcal extract. Effect of PRPP concentration. The assay was performed as described in Methods with 25 µl extract containing 0.22 mg protein.

Effect of substrate concentrations and requirements for Mn^{++} and PRPP Fig 4 and 5 show the rate dependence of adenine phosphoribosyltransferase on the adenine and PRPP concentrations. Maximum velocity of the enzyme reaction seems to be reached near a concentration of adenine of 0.3 mM and of PRPP at a concentration of 5 mM under the experimental conditions used. The adenine phosphoribosyltransferase activity has an absolute requirement for Mn^{++} and PRPP being negligible in crude extracts without Mn^{++} and PRPP and showing no activity after dialysis for 22 hours without Mn^{++} or without PRPP (Table 4)

Stability of the enzyme Adenine phospho-

TABLE 4 *Adenine Phosphoribosyltransferase Activity in Cell-free Extracts from N meningitidis*

Expt. No.	State of extract	Protein mg/assay	Cation added	Substrate added	C-adenine converted nanomoles	Per cent activity
1	Crude-new	0.12	Mn^{++}	PRPP	0.78	100
	*		None	PRPP	0.05	6.4
			Mn^{++}	None	0.02	2.5
			None	None	0.01	1.3
2	Crude new	0.28	Mn^{++}	PRPP	2.42	100
	Dialyzed 3 hours		Mn^{++}	PRPP	1.58	65
	Dialyzed 22 hours		Mn^{++}	PRPP	0.68	28
			None	PRPP	0	0
			Mn^{++}	None	0	0
	Kept frozen t −20 C for 8 days		Mn^{++}	PRPP	2.06	85

Experimental conditions are as described in Methods. * Extract dialyzed against 100 olumes of Tris/HCl buffer 0.05 M pH 7.6 at 4 C.

TABLE 3 *Labelling of Purine Metabolites from ^{14}C-8-adenine in Intact Cells*

Organism	Time of incubation sec	Incorporation calculated as picomoles adenine					
		A	Hx	AR	I	AMP+ADP +ATP	GMP+GDP +GTP
N meningitidis							
Strain M1	15	20.8	–	–	–	124.6	3 2
	60	10.2	–	–	–	191.5	6 7
	120	5.0	–	–	–	234.0	8 0
E. coli K12	15	54 0	30.3	23.3	4 4	613.9	73 4

The assay was as described in Methods.

Medium A the growth is only 82 per cent with 2 aminopurine in a concentration of 1×10^{-3} M (19) Fig 2 shows the inhibition of growth of strain M1 in Medium KC devoid of adenine and guanine with 2,6-diaminopurine and 6-mercaptopurine, both in the concentration of 2.1×10^{-3} M. There is a pronounced effect on the growth of *N meningitidis* with both analogues. This points to active adenine and guanine-hypoxanthine phosphoribosyltransferases in *N meningitidis* (22, 25)

Labelling of purine metabolites from ^{14}C 8-adenine The labelling of AMP ADP and ATP from ^{14}C-8-adenine added to cells of *N meningitidis* strain M6 has been demonstrated earlier (17) A re-examination of the labelling from ^{14}C-8-adenine was performed with *N meningitidis* strain M1 *E. coli* was simultaneously examined to provide a means for comparison with better known systems (11) Table 3 shows that adenine also in strain M1 of *N meningitidis* is incorporated into AMP ADP and ATP whereas no label from adenine appeared in adenosine, hypoxanthine or inosine. A faint labelling of the guanosine 5-nucleotides GMP GDP and GTP also occurred. In *E. coli* adenine is rapidly incorporated into the adenosine 5' nucleotides and guanosine 5-nucleotides The spots corresponding to adenosine, hypoxanthine and inosine also showed radioactivity (Table 3)

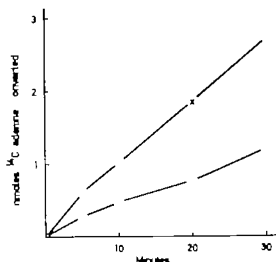


Fig 3 Activities corresponding to adenine phosphoribosyltransferase in crude meningococcal extract. Experiment A (×——×) was performed with 25 µl extract containing 0.29 mg protein. In experiment B (●——●) half as much extract was used as in experiment A. Experimental conditions as described in Methods

Adenine Phosphoribosyltransferase

Effect of enzyme concentration and time Fig 3 shows the enzyme activity related to time. In experiment B, half as much extract was used as in experiment A Each point represents the sum of the adenosine 5-nucleotides formed (AMP + ADP + ATP) because of an active AMP and ADP kinase present in the crude extracts. The kinases require phosphate donors and divalent cation for activity Phosphate donors are obviously present in crude extracts, and both Mn^{++} and Mg^{++} may be used as cations in the reactions, as found in other systems (31 33)

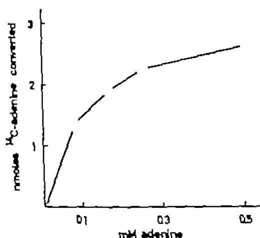


Fig 4 Activities corresponding to adenine phosphoribosyltransferase in crude meningococcal extract. Effect of adenine concentration. The assay was performed as described in Methods with 25 µl extract containing 0.22 mg protein.

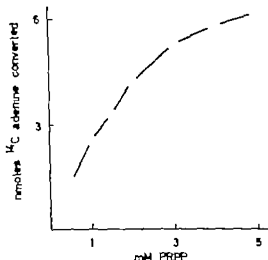


Fig 5 Activities corresponding to adenine phosphoribosyltransferase in crude meningococcal extract. Effect of PRPP concentration. The assay was performed as described in Methods with 25 µl extract containing 0.22 mg protein.

Effect of substrate concentrations and requirements for Mn^{++} and PRPP Fig 4 and 5 show the rate dependence of adenine phosphoribosyltransferase on the adenine and PRPP concentrations. Maximum velocity of the enzyme reaction seems to be reached near a concentration of adenine of 0.5 mM and of PRPP at a concentration of 5 mM under the experimental conditions used. The adenine phosphoribosyltransferase activity has an absolute requirement for Mn^{++} and PRPP being negligible in crude extracts without Mn^{++} and PRPP and showing no activity after dialysis for 22 hours without Mn^{++} or without PRPP (Table 4)

Stability of the enzyme Adenine phospho-

TABLE 4 *Adenine Phosphoribosyltransferase Activity in Cell-free Extracts from* N meningitidis

Expt No.	State of extract	Protein mg/assay	Cation added	Substrate added	^{14}C-adenine converted nanomoles	Per cent activity
1	Crude-new	0.12	Mn^{++}	PRPP	0.78	100
	"		None	PRPP	0.05	6.4
			Mn^{++}	None	0.02	2.5
			None	None	0.01	1.3
2	Crude new	0.28	Mn^{++}	PRPP	2.42	100
	Dialysed 5 hours*		Mn^{++}	PRPP	1.58	65
	Dialysed 22 hours*		Mn^{++}	PRPP	0.68	28
			None	PRPP	0	0
			Mn^{++}	None	0	0
	Kept frozen at −20° C for 8 days		Mn^{++}	PRPP	2.06	85

Experimental conditions are as described in Methods. * Extract dialysed against 100 volumes of Tris/HCl buffer 0.05 M pH 7.6 at 4° C.

ribosyltransferase activity in crude meningococcal extract in Tris/HCl buffer (0 05 M – pH 7 6) is very unstable to heat treatment (Fig 6) After exposure to 45 C for 3 minutes the activity was only about 50 per cent, and no activity remained after similar treatment at 65 C.

Table 4 shows that the activity of the enzyme falls to 65 and 28 per cent, respectively after dialysis of the extract for 3 and 22 hours at 4 C against Tris/HCl buffer Freezing of the crude extract for 8 days resulted in an activity of 85 per cent.

Effect of buffers and pH on the activity The best defined pH curve was obtained with potassium phosphate buffer and Mn^{++} (1 mM) as the cofactor the pH optimum being at 7 4 (Fig 7) In K phosphate buffer with Mg^{++} (10 mM) as the divalent cation, the activity of adenine phosphoribosyltransferase showed a fairly broad optimum between pH 7 4 and 8 0 (Fig 8) In Tris/HCl buffer the activity was low being only 22 per cent with Mn^{++} (1 mM) at pH 7 4 and 32 per cent with Mg^{++} (10 mM) at pH 7.8 compared to

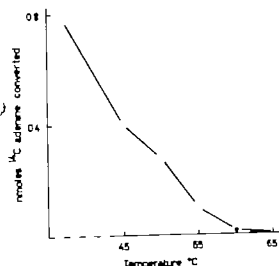


Fig 6 Activities corresponding to adenine phosphoribosyltransferase in crude meningococcal extract. Portions of 0.6 ml extract prewarmed to 37 C, were placed in a water bath at the temperatures indicated for 3 minutes, chilled in an ice-water bath, and examined for activity The assay was performed as described in Methods with 25 µl extract containing 0.15 mg protein.

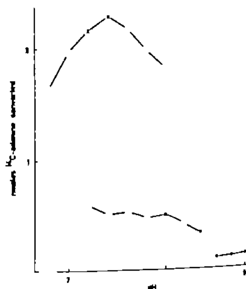


Fig 7 Activities of adenine phosphoribosyltransferase in K-phosphate (×—×) Tris/HCl (●—●) and glycine/KOH buffer (O—O). The cation was Mn^{++} (1 mM) and 25 µl extract contained 0.28 mg protein. The rest of the procedure as described in Methods. The pH values are those given by the buffers at 37 C.

that with K phosphate buffer With glycine/KOH buffer at pH 8.6 to 9 0 the activities were very low with both cations (Fig 7 and 8)

Effect of divalent cations. Stimulation of the adenine phosphoribosyltransferase reaction rose with increasing divalent cation concentration and reached its maximum at Mn^{++} levels of 0.5 to 1.5 mM when K phosphate buffer pH 7 4 and 0.5 mM PRPP were used (Fig 9) The Mg^{++} concentration had to be from 5 to 20 mM for maximum activity in K-phosphate buffer pH 7.8 with 0.5 mM PRPP (Fig 10) Ca^{++} at 1 mM had no effect (Table 5) Mn^{++} is a far better stimulator of the activity than Mg^{++} Under otherwise identical conditions, 1 and 10 mM Mg^{++} showed 18 and 32 per cent activity respectively compared with 1 mM Mn^{++} (Table 5) 1 mM Mg^{++} even showed an inhibitory effect together with 1 mM Mn^{++} the activity being 80 per cent (Table 5)

Effect of sulphhydryl reagents and product inhibition. Three sulphhydryl reagents, iodoacetate, o-iodobenzoic acid and parachloro-

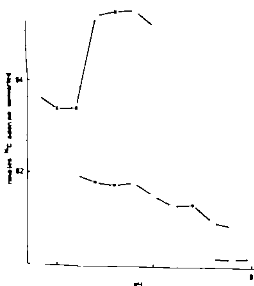


Fig 8 Activities of adenine phosphoribosyltransferase in K-phosphate (×—×) Tris/HCl (●—●) and glycine/KOH buffer (○—○). The cation was Mg^{++} (10 mM) and 25 µl extract contained 0.26 mg protein. The rest of the procedure as described in Methods. The pH values are those given by the buffers at 37 C.

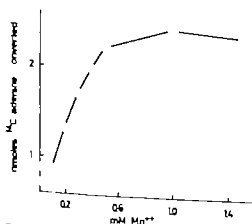


Fig 9 Activities of adenine phosphoribosyltransferase in crude extract from *N meningitidis* with increasing Mn^{++} concentration. The procedure was as described in Methods with 25 µl extract containing 0.26 mg protein.

mercuribenzoate showed no inhibitory effect in the concentrations used (Table 6). The product inhibition of pyrophosphate was strong the activity being only 39 and 2.5 per cent with 1 and 10 mM PP respectively.

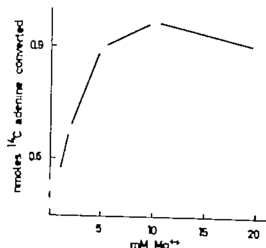


Fig 10 Activities of adenine phosphoribosyltransferase in crude extract from *N meningitidis* with increasing Mg^{++} concentration. The procedure was as described in Methods with 25 µl extract containing 0.29 mg protein.

TABLE 5 *Activity of Adenine Phosphoribosyltransferase in Crude Extract from* N meningitidis *with Different Cations*

Cations	Concentration of cations mM	^{14}C-adenine converted nanomoles	Per cent activity
Mn^{++}	1	3.00	100
Mg^{++}	1	0.56	18
Mg^{++}	10	0.96	32
Mn^{++} and	1		
Mg^{++}	1	2.40	80
Ca^{++}	1	0.22	7
None	–	0.25	8

The enzyme activity was assayed in K-phosphate buffer pH 7.8 and with 25 µl extract containing 0.29 mg protein. The rest of the procedure as described in Methods.

AMP also showed inhibitory effect. The activities were found to be 95 and 74 per cent with 1 and 10 mM AMP under the experimental conditions used (Table 6).

Search for Other Enzyme Functions Involved in the Utilization of Adenine

Purine-nucleoside phosphorylase Activities corresponding to this enzyme could not be demonstrated in extracts from *N meningitidis*

TABLE 6. *Effect of Sulphhydryl Reagents and Enzyme End Products on Adenine Phosphoribosyltransferase Activity in Crude Extracts from* N meningitidis

Expt. No.	Protein mg/assay	Chemical added	Final conc. of chemical mM	^{14}C-adenine converted nanomoles	Per cent activity
1*	0.29	None	–	2.73	100
	"	Iodoacetate	0.1	2.82	100
		Iodoacetate	1.0	2.84	100
	"	IBA	0.1	2.88	100
		IBA	1.0	2.77	100
		PCMB	0.1	2.85	100
		Na–PP	1.0	1.62	59
	"	Na–PP	10.0	0.07	2.5
2**	0.22	None	–	9.91	100
		AMP	1.0	9.49	95
		AMP	10.0	7.33	74

* The experimental procedure as described in Methods. ** In this experiment 0.025 µmole ^{14}C-8-adenine (10 µCi/µmole) and 0.1 µmole PRPP were used. The rest of the procedure was as described in Methods. Abbreviations: IBA = o-iodobenzoic acid, PCMB = parachloromercuribenzoate. Both chemicals were dissolved in KOH and neutralised by HCl. Iodoacetate was used as the potassium salt.

TABLE 7 *Activity of Purine-nucleoside Phosphorylase in Crude Dialysed Extracts from* N meningitidis *and* E. coli

Organism	Protein mg/assay	Time of incubation min	^{14}C-adenine converted nanomoles**	Enzyme activity nanomoles adenine converted per mg protein at the time indicated
N meningitidis	0.07	30	–	–
Strain M1	0.04*	30	–	–
E. coli K12	0.007	5	10.8	1542
	0.006*	5	12.1	2016

Assay as described in Methods. * This bacterial culture was grown with 2 mM adenosine with a view to allowing induction of the enzyme (6) as described in Methods. ** The sum of the adenosine, inosine and hypoxanthine formed by the *E. coli* extracts.

with adenine and ribose 1 phosphate as substrates. *E coli* extracts were highly active in the same technique. Even extracts containing only one tenth as much protein as those used in experiments with *N meningitidis* showed strong activity (Table 7). Purine-nucleoside phosphorylase is reported to be inducible in *E. coli* by adenosine (6). Table 7 shows that induction was achieved in *E coli* whereas the extract from *N meningitidis* showed no activity. The adenosine formed in the *E. coli* experiments was partly converted to inosine and hypoxanthine.

Adenine deaminase. An active adenine deaminase could not be demonstrated in crude extracts from *N meningitidis*. When recording the spectra from 240 to 280 nm of the reaction mixture (see Methods) there was no change in the absorption spectrum. A control with crude dialysed extract run with radioactive adenine in Tris/HCl buffer pH 7.2, and chromatography of the supernatant and ratio-

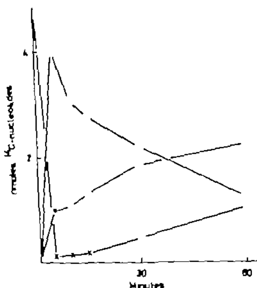


Fig 11 Variation of the radioactivity of AMP (×—×) ADP (●—●) and ATP (○—○) during incubation of crude meningococcal extract with ¹⁴C-AMP ATP and Mg^{++} (see Methods) 5 µl extract with 0.16 mg protein was used in the assay

radiography showed only one spot on the film, corresponding to adenine.

Adenylate kinase This enzyme seems to be very active in *N meningitidis* and there is also a rapid conversion of ADP to ATP This reaction is the result of an active nucleoside-diphosphate kinase EC 2.7 4 6 (4) Fig 11 shows that nearly all the ¹⁴C-AMP is converted into radioactive ADP and ATP within 5 minutes in the presence of ATP and Mg^{++} After 5 minutes there is also a gradual interconversion of the nucleotides.

DISCUSSION

The mutant M1 *his pro A G* (6-1b) of *N meningitidis* can grow with adenine plus guanine or xanthine. Hypoxanthine can be utilized as the only purine base (Table 2) As shown, *N meningitidis* has an active adenine phosphoribosyltransferase, and the growth requirements point also to active guanine-hypoxanthine and xanthine phosphoribosyltransferases. That this is the case has actually been demonstrated (*S Jyssum* to be published)

Among the purine nucleosides, only adenosine seems to be metabolized by *N meningitidis* (Table 2) Adenosine may be broken down to adenine either by a purine-nucleoside phosphorylase or a nucleosidase (32) The adenine formed is then converted to AMP by adenine phosphoribosyltransferase.

The cleavage of the glycosidic bond of adenosine in *N meningitidis* seems to be the result of a direct hydrolysis (*S Jyssum* to be published) An adenosine kinase does not seem to be present in *N meningitidis* (*S Jyssum* to be published)

The mutant cannot utilize any of the monoribonucleotides for growth (Table 2) This is consistent with the fact that *N meningitidis* cannot utilize thymidine 5'-monophosphate (TMP) for growth even if an active TMP kinase is present in the organism (17)

The growth of the prototrophic strain M1 of *N meningitidis* is strongly inhibited by 2 6-diaminopurine and 6-mercaptopurine (Fig 2) These purine analogues are reported to be substrates for the phosphoribosyltransferases, the analogues being converted to their respective ribonucleotides. In most cases, this action is a prerequisite for providing an inhibitory form of the analogue (3 22) The inhibitory effects of the analogues are therefore consistent with the presence of active adenine and guanine hypoxanthine phosphoribosyltransferases in *N meningitidis*

The radioactive metabolites found in the ethanolic extracts from whole cells of *N meningitidis* and *E coli* after growth with ¹⁴C-8-adenine points to fundamental differences in the metabolism of this purine base in the two species. In *N meningitidis* adenine seems to be utilized for direct incorporation into AMP only (Table 3) *E. coli* contains purine-nucleoside phosphorylase which may convert adenine to adenosine (10, 32) Adenosine is deaminated to inosine (23) which in turn is converted to hypoxanthine by the purine-nucleoside phosphorylase. Adenine and hypoxanthine may subsequently be converted to the nucleotide level by condensation with PRPP catalyzed by the phosphoribosyltransferases (Fig 1) (9 25)

Adenine phosphoribosyltransferase described by *Kornberg et al.* in 1955 (24) has since then been found in a variety of microorganisms (2, 9, 22, 25, 34) and in mammalian systems (14, 36).

Fig. 3 shows the amount of product formed (AMP + ADP + ATP) by adenine phosphoribosyltransferase from *N. meningitidis* as a function of time and amount of enzyme. The activity of the enzyme is dependent on the concentration of the substrates at least up to 0.5 mM for adenine and 5 mM for PRPP under otherwise identical conditions (Fig. 4 and 5).

The enzyme from *N. meningitidis* is quite unstable in Tris/HCl buffer. It loses 15 per cent of the activity after storage at −20° C for 8 days (Table 4). This is in contrast to the enzyme in crude extracts from *E. coli*, which is reported to be stable in Tris/HCl buffer after storage at −15° C for many months, and even after dialysis (34). Adenine phosphoribosyltransferase from *Mycoplasma mycoides* loses activity on freezing and thawing (34) similar to the enzyme from *N. meningitidis*. Purified enzyme from *E. coli* was found to be unstable to storage at −20° C but this could be prevented by storage in K phosphate buffer (9).

Heating of the *N. meningitidis* enzyme for 3 minutes at 60° C resulted in a loss of 98 per cent of the activity (Fig. 6). If crude extracts of *E. coli* and *Lactobacillus casei* were incubated at 60° C for 5 minutes, the loss was about 96 and 77 per cent, respectively (25).

The highest activity of adenine phosphoribosyltransferase in crude extracts from *N. meningitidis* was found in phosphate buffer at pH 7.4 with Mn^{++} as divalent cation and in a concentration 1–3 times that of PRPP (Fig. 7 and 9). If Mg^{++} was used as cation the highest activity was at pH 7.8 in phosphate buffer and with Mg^{++} at concentrations 10–40 times the PRPP concentration of 0.5 mM (Fig. 8 and 10). Using purified enzyme from *E. coli* maximum activity was found in Tris/HCl buffer in the vicinity of pH 8.0. With phosphate buffer the activity at pH 7.8 was 90 per cent of that observed with Tris/HCl buffer. The *E. coli* enzyme had an absolute requirement for divalent cation which could be satisfied either by Mn^{++} or Mg^{++}. $MnCl_2$ was slightly better than $MgCl_2$ and both had their optimum at 1 to 2 times the PRPP concentration used in the experiments. Calcium was able to inhibit the activity significantly (9). Optimal activity of the purified enzyme from *Bacillus subtilis* was in Tris buffer at pH 8.29 and with Mg^{++} as cation (2). Here calcium supported the reaction at a maximum rate of only 5 per cent of that with magnesium (2). Ca^{++} at 1 mM had no effect on the activity with extract from *N. meningitidis* (Table 5). The far higher activity of adenine phosphoribosyltransferase from *N. meningitidis* in phosphate buffer compared to that in Tris/HCl buffer might point to a protection against enzyme inactivation as found by purified enzyme from *E. coli* (9). Adenine phosphoribosyltransferase from human blood platelets and Ehrlich ascites tumour cells (13, 14) is reported to be more active with Mn than with Mg^{++} as cation, as found with the enzyme from *N. meningitidis*.

Adenine phosphoribosyltransferase from *N. meningitidis* seems not to contain active SH-groups since the sulphhydryl blockers iodoacetate, iodobenzoic acid and para-chloromercuribenzoate had no effect on the activity (Table 6). The activity of the enzyme from *E. coli* (9), *Mycoplasma* (34) and from human blood platelets (13) are reported to be unaffected by mercaptoethanol, dithiothreitol and cystein, respectively. *B. subtilis* enzyme did not react on PCMB but was inhibited 100 per cent by 0.1 mM iodoacetate (2).

Both products, AMP and PP are found to be inhibitory to the adenine phosphoribosyltransferase—catalyzed reaction in *N. meningitidis* (Table 6). This is consistent with the findings in other systems (2, 9, 13, 34).

The results show that *N. meningitidis* extracts have activities corresponding to the enzyme adenine phosphoribosyltransferase. The heavy labelling of the nuclei acids of

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HYALURONIDASE AND AMINOPEPTIDASE ACTIVITY IN CULTURES OF *STREPTOCOCCUS MITIS*, ATCC 903

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The formation and release of hyaluronidase and aminopeptidase in cultures of *Streptococcus mitis* strain ATCC 903 was studied by anaerobic cultivation in a stirred fermentor at constant pH. The nutrient medium was a glucose-proteose-peptone broth, glucose being the growth limiting factor More than 95 per cent of the hyaluronidase and more than 85 per cent of the aminopeptidase was liberated during a period of rapid autolysis following the exhaustion of glucose. A good correlation could be demonstrated between the degree of autolysis and the release of hyaluronidase, indicating the cell-bound character of the enzyme. The results also indicate the possible use of aminopeptidase as a marker for intracellular products.

Hyaluronidase production has been demonstrated in streptococci, clostridia, staphylococci, pneumococci and corynebacteria. Most earlier investigations support the opinion that streptococcal and staphylococcal hyaluronidases are liberated into the medium during active growth (13 14) *Meyer et al* (8) found hyaluronidase activity in culture filtrates and no enzyme in extracts from cells of *Clostridium welchii*. *Rogers* (13) found that in some cultures of *Cl. welchii* most of the enzyme release took place while the organisms were autolysing. Inconclusive results concerning the release of hyaluronidase in earlier investigations may be due to variations in the absence of control of pH during growth.

Conclusions regarding the release mechanism of enzymes from bacterial cells cannot be based solely on quantitation of the enzymes in the extracellular medium during the growth cycle. Measurement of cell lysis or cell damage, which may be insufficient to cause measurable decrease in cell density should be made, preferably by the use of an intracellular marker whose presence in the extracellular medium is proportional to the extent of cell damage under all experimental conditions. *Pollock* (12) used α-glucosidase as an intracellular marker in studies of the release of penicillinase from *Bacillus subtilis*. Aminopeptidase activity in culture supernatants has been used as an indicator of bacterial lysis in an investigation into proteinase formation by a strain of *Micrococcus* by *McDonald* (6) Furthermore, leucine aminopeptidase of *Escherichia coli* has been found located inside the membrane barrier of intact cells by *Neu & Heppel* (9) and by *Simmonds & Toye* (16)

The aim of the present investigation was to study the occurrence of hyaluronidase in

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the growth medium of *Streptococcus mitis* during anaerobic growth at controlled pH. Rapid autolysis took place after the exhaustion of glucose. The increase in hyaluronidase and aminopeptidase activities in the culture medium was correlated to the degree of autolysis. The results indicate that hyaluronidase is a cell-bound enzyme in *Streptococcus mitis* and that it is released from the bacteria by an autolytic process. The results also indicate that aminopeptidase may be a sensitive indicator of cell lysis.

MATERIALS AND METHODS

Strain: Streptococcus mitis (ATCC 903) Preliminary experiments showed that after repeated replatings on blood-agar plates the yields of hyaluronidase in liquid media decreased. Therefore, the cells of one ampoule were cultured once in the medium described below and lyophilized in several ampoules. A fresh ampoule was used in each experiment.

Media Except for some preliminary experiments, a medium called PP1 was used. It had the following composition proteose-peptone (Difco) 40 g, $Na_2HPO_4 \cdot 2H_2O$ 1.0 g, KH_2PO_4 0 4 g, glucose 10 g (if not stated otherwise) Ca-panthothenate 1.2 mg, nicotinic acid 1.2 mg pyridoxine-HCl 1.2 mg, thiamine-HCl 1.2 mg, pimelic acid 1 2 mg, riboflavine 0 12 mg, $MgSO_4 \cdot 7H_2O$ 20 mg, $MnSO_4 \cdot 4H_2O$ 10 mg $FeSO_4 \cdot 7H_2O$ 6 mg citric acid 6 mg, distilled water to a final volume of 1 l. The vitamins and the trace elements were each heat sterilized separately in 100-fold concentrated solutions. The bulk of the medium was sterilized at 121 C for 15 minutes. The medium used for precultures had an increased buffer capacity the amount of $Na_2HPO_4 \cdot 2H_2O$ being 7.5 g/l and KH_2PO_4 3.0 g/l. The glucose concentration was 5 g/l

Cultivation technique The cells of one ampoule were transferred to two 50-ml flasks with tight caps, each containing 50 ml preculture medium. The flasks were incubated 10–15 hours at 37 C. The precultures were used as inocula for a 1 l culture in a stirred fermentor (FG 500 Biotec, Stockholm, Sweden) The pH of the culture was kept at 6.5 by automatic titration with a titrator (TTT1 Radiometer Copenhagen, Denmark) and an automatic burette (Abu 12, Radiometer) The titrant was a 5M sterile sodium hydroxide (Titrisol) The temperature was regulated at 37 ± 0 2 C. The impeller speed was 200 rev/min. Sterile nitrogen gas was passed through the medium at a flow rate of 0.15 l/min. Samples of 10 or 20 ml were taken at regular intervals from the culture and centrifuged at 20,000 × g for 10 min at 4° C. The supernatant fluid was used for determination of hyaluronidase and aminopeptidase activity and *for the determination of the glucose concentration*. The pellet was used for determination of bacterial growth.

Determination of enzymatic activities: Hyaluronidase activity was determined by the viscosimetric method described by *Hultin* (3, 4) A mixture consisting of 3 0 ml of a solution of 0 2 per cent sodium hyaluronate and 0.2 per cent NaCl dissolved in 0 1M sodium acetate-acetic acid buffer pH 5.5 and 1.0 ml of the sample supernatant fluid was incubated in an Ostwald viscosimeter at 37 C. The change in viscosity was calculated from the outflow time of the mixture. The hyaluronidase activity was calculated from the formula of *Hultin* (4) and expressed as Hultin units (H U). Aminopeptidase activity was determined by a modification of the colorimetric method described by *Goldberg & Rutenburg* (2) and expressed in units (U) The accuracy of this modified method by which to measure aminopeptidase activity in cell-free extracts of this strain has been investigated by *Linder et al.* (5) In this work, the method is described in detail. In the present investigation, the determination of aminopeptidase activity was made against a blank substituting sterile medium for the culture supernatant.

Materials Hyaluronic acid grade III-S and reagents for the determination of aminopeptidase activity were obtained from Sigma Chem Co St. Louis, Mo., U.S.A. and Trypticase from B. D Merieux, Marcy-L étoile, Rhône, France.

Determination of the glucose concentration. The glucose concentration of the sample supernatant was determined by the glucose-oxidase method (Glox, AB KABI Stockholm, Sweden)

Determination of bacterial growth After centrifugation of the culture sample, the cells were washed once in 10 ml cold (4–7 C) 0.01 M phosphate buffer pH 6.8 and again centrifuged The cells were then completely resuspended in 20 ml distilled water by the aid of a tissue homogenizer (A. H. Thomas, Philadelphia, U.S.A.) The optical density of an adequately diluted sample of the suspension was immediately determined in Zeiss spectrophotometer (PMQ II) at 550 nm (A_{550}). The dilutions were made so that all readings were made in the range 0 100–0.800.

The average size of the cells may vary considerably from one phase of the growth cycle to another and as comparisons are made in this work between cells of logarithmic and autolytic phases of growth, the relation between optical density and another estimate of growth had to be determined for cells of different phases of the growth cycle. The relation between optical density and the dry weight of the cells was determined in the following way. Samples of 320 ml were taken at different phases of growth

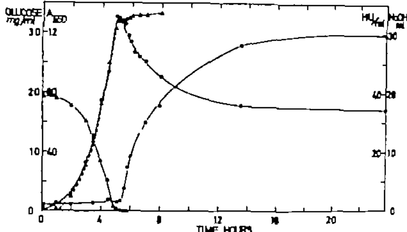


Fig 2 Relationship between bacterial concentration, glucose concentration, acid production and hyaluronidase activity in the culture medium. Bacterial concentration (A_{650}) ○——○, glucose concentration (mg/ml) ▲——▲, titrant used (ml 5M NaOH) △——△ hyaluronidase activity (H.U./ml) ●——● It should be observed that the cell yield was approximately proportional to the initial concentration of glucose cf Fig 1

of titrant used were parallel in the exponential phase. This information was of great value in subsequent experiments with this strain since the consumption of titrant could be taken as an estimate of growth.

Liberation of hyaluronidase and aminopeptidase The activities of both enzymes that occurred in the culture medium at the beginning of the cultivation originated from the inocula. The amounts of the enzymes released during logarithmic growth were insignificant compared to the release that started upon glucose exhaustion (Fig 1 and 2)

The beginning of the period of rapidly increasing hyaluronidase and aminopeptidase activity in the extracellular medium coincided in time closely with the exhaustion of glucose and also with the beginning of a period of decreasing cell turbidity The relationship between autolysis, expressed as decrease in optical density (ΔA_{650}) and hyaluronidase activity of the culture fluid is shown in Fig 3 The hyaluronidase activity of the culture medium increased proportionally to bacterial lysis when the cultivations were performed in media with an initial glucose concentration of 20 mg/ml, while the parallelism between hyaluronidase activity and cell lysis was not equally consistent during the whole phase of cell lysis in cultivations with an initial glucose concentration of 10 mg/ml. This relationship between the increase in enzyme activity and the decrease in optical density of the cultures was practically the same for hyaluronidase and aminopeptidase. The increase in hyaluronidase activity per unit decrease in cell density (ΔA_{650}) in cultures grown in 10 mg/ml of glucose was about twice that in media of 20 mg/ml of glucose (Fig 3) No such difference in specific increase in aminopeptidase activity of cultures grown in different glucose concentrations was observed.

DISCUSSION

The observation that the cell density rapidly decreased upon glucose depletion is in agreement with the observation by *Shockman et al* (15) on lysis of *Streptococcus faecalis*. These authors suggested that lysis may follow the depletion of glucose the presumptive precursor of cell wall polysaccharides as well as the energy source

The increase in hyaluronidase and aminopeptidase activity of the extracellular medium during the autolysis following glucose exhaustion may be explained by a number of hypo-

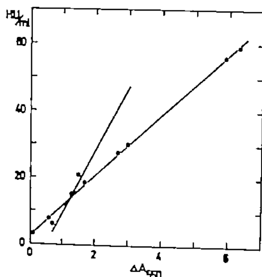


Fig 3 Relationship between the degree of autolysis and hyaluronidase activity in the culture medium. The values were obtained from the experiments described in Figs. 1 and 2. Hyaluronidase activity (H.U./ml) plotted against decrease in optical density (ΔA_{550}) Data obtained from the cultivation performed in medium containing 10 mg/ml of glucose (see Fig. 1) ●——● Data obtained from the cultivation performed in medium containing 20 mg/ml of glucose (see Fig. 2) ○——○.

theses. 1 The formation of the enzymes takes place during exponential growth. The enzymes are cell-bound and released by autolysis. 2. The synthesis of the enzymes is repressed by glucose. Upon depletion of glucose the synthesis of the enzymes starts and they are released by autolysis or from intact cells at a rate equal to the rate of autolysis. It is well-known that the rate of synthesis of many enzymes is reduced by glucose and also by other energy-yielding substances. This phenomenon, termed glucose repression, was established by *Epps & Gale* (1) and later renamed catabolite repression by *Magasanik* (7) The results of the present investigation are in agreement with the possibility that both enzymes are synthesized at an increased rate upon the depletion of glucose and at the same time liberated by autolysis of a fraction of the cells. 3. The enzymes are synthesized during the whole growth cycle. The activity of the preformed enzyme molecules is inhibited by glucose. Upon depletion of glucose, active enzymes are liberated. This hypothesis is based on the phenomenon called catabolite inhibition which term was defined by *Paigen & Williams* (10) No definite conclusions regarding the synthesis and release of the enzymes can be drawn until the cell-bound fractions of the enzymes have been studied by disintegration of cells from different phases of the growth cycle In two subsequent articles, the extraction of cell bound hyaluronidase and aminopeptidase by disintegration of cells and comparisons between cell-bound and released enzymes in different phases of growth will be reported.

The observation that the amount of hyaluronidase liberated per unit decrease in cell density in cultures of an initial glucose concentration of 10 mg/ml was approximately twice that in cultures grown in 20 mg/ml of glucose may be explained by exhaustion of other medium components necessary for protein synthesis during the extended growth permitted by the higher concentration of the energy source (see Fig. 2)

Peptidases play an important role in bacterial metabolism. The hydrolysis of peptides to amino acids by intracellular peptidases provides the cell with the necessary building blocks for protein synthesis and also facilitates the diffusion of peptides into the cell (11) In this investigation the aminopeptidase of *Streptococcus mitis* was studied primarily as an indicator of autolysis. The use of aminopeptidase as an intracellular marker will be the subject of further studies.

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A few strains were isolated from blood, sputum, ear secretion, vaginal secretion or faeces. The majority of specimens were collected from three hospitals in the city of Malmö. In addition 10 other hospitals in southern Sweden served by the laboratory were represented. In the primary material many isolates originated from the same ward and even from the same patient. The strains used for extensive biochemical investigation were selected in such a way that each ward would not be represented more than once by a particular biotype. The *P rettgeri* strains used for comparative purposes came from the same geographical area and from the same kinds of specimen.

The strains selected represented four main biogroups: *Proteus inconstans A (Providencia alcalifaciens) 8 strains*. *Proteus inconstans B (Providencia stuartii)* 83 strains an intermediate group showing features of *Proteus inconstans B* but with positive urease reaction, *14 strains*. *Proteus rettgeri*, 18 strains. An additional three strains were examined, designated 70141 G 82 and 103 which were aberrant in their biochemical reactions.

The strains were kept as stab cultures on meat extract agar at room-temperature or lyophilized.

Methods

As inoculum was used an overnight human blood agar culture. Unless otherwise specified the incubation temperature was 35 C. The tests were read after 1 2, 4 and 7 days. In some cases they were submitted to prolonged observation.

Motility Nutrient agar stab culture. Agar content 0.2 per cent.

Oxidase Freshly prepared solution of 0.3 per cent tetramethyl-p-phenylenediamine dihydrochloride in 30 per cent ethanol. The test was performed on filter paper according to *Kovács* (15)

Citrate Two methods were used *Koser's* citrate modified (6) and *Simmons'* citrate agar (commercial BBL)

KCN Møller's method modified (7)

Nitrate reduction. According to *Cowan & Steel* (6)

Malonate and phenylalanine tests. Combined medium according to *Shaw & Clarke* (22)

Hyd gen sulphide SIM medium (commercial BBL)

Voges-Proskauer test. Barritt's method modified (17) Incubation at room temperature (22–24 C)

Beta galactosidase According to *Bulow* (*) Readings after 20 minutes and 3 hours.

Indole The method of *Kovács* (14) was made more sensitive by adding a drop of xylene and shaking vigorously before adding *Kovács'* reagent (13) The medium used was meat extract with 1 per cent Bacto tryptone (Difco)

Decarboxylase tests. Møller's method modified (6)

Gelatin liquefaction. Nutrient gelatin according to *Cowan & Steel* (6) Inoculated tubes are incubated at 35 C overnight to promote growth. From the second day onwards they were kept at room temperature.

Esculin hydrolysis According to *Cowan & Steel* (6)

Acid formation from carbohydrates. Liquid medium according to *Nilsén* (17) with 1 per cent of the test substance. Durham tubes were used for detection of gas.

Urease tests. Three methods were used, all tubes being heavily inoculated and incubated at 35° C. 1) The weakly buffered fluid medium described by *Jessen* (9) The tubes were read after 1, 4 and 24 hours. The same medium without urea served as a control. The reaction was considered positive if a red colour corresponding to pH 6 6–6.7 appeared in 4 hours. 2) The strongly buffered urea broth according to *Rustigian & Stuart* (commercial BBL) The test was read as above and tubes without urea were used as a control. The reaction was considered positive if the colour change developed in 24 hours 3) *Christensen's* urea agar (commercial BBL) in slanted tubes which were read after 4 24 and 48 hours. The reaction was considered positive if the colour change in 24 hours extended over the entire slant and began to penetrate the butt.

RESULTS

General Characteristics

All strains grew well on ordinary media and produced smooth, glistening colonies. As regards the *P inconstans* strains, 22 out of 91 were recorded as non-motile. Motility, if present, was often very sluggish, which is in accordance with earlier observations (8, 18). All strains were oxidase negative gram negative rods that fermented glucose rapidly with or without a small amount of gas. They all reduced nitrate to nitrite and had a strong phenylpyruvic acid reaction.

Urease Reactions

The three methods showed good agreement for every strain. The reactions of the intermediate strains were generally slower than those of the average *P rettgeri*. The typical *P inconstans* showed no colour change in any test even after incubation for 2 days. One of the aberrant strains (G 82) was positive.

three strains showing late fructose fermentation two were mannose negative. These strains were isolated in two different hospitals, but one of the patients proved to have been treated in both. The third strain was also sorbose positive and indole negative. It was isolated from faeces from a patient who had contracted diarrhoea during a visit to North Africa.

All *P inconstans B* strains produced acid from galactose and trehalose but were erythritol negative.

The intermediate group could be distinguished from *P inconstans B* only by its ability to hydrolyse urea.

The *P rettgeri* strains were all erythritol positive and galactose positive. In addition, all the strains but one fermented arbutin rapidly the negative strain being one of the two that did not ferment arabitol. All *P rettgeri* isolates were trehalose negative.

The biochemical reactions of the aberrant strains will be discussed below

DISCUSSION

The small collection of *P inconstans A* isolates exhibited no unusual features. The negative galactose reaction, however is of some interest. It was described by *Stuart et al.* (25) to apply to *Paracolon* Type 29911 which corresponds to a maltose positive variant of *P inconstans A* Authors who have later studied the galactose reaction of *P inconstans* (3 10 23) have made no distinction between the two main biotypes. As galactose may have a differential diagnostic value for the identification of *P inconstans A* a study of this reaction in a larger number of strains would obviously be of interest.

The majority of *P inconstans B* strains conformed to the commonly recognized biochemical pattern of this organism (8 19 24) Most of the aberrations have been reported by several authors. Esculin hydrolysis, however seems to have been found only by *Singer & Bar-Chay* (23) Late gelatin liquefaction has been reported by *Edwards & Ewing* (7)

Fermentation of mannose and sorbose has previously been studied only by *Kauffmann* (10) who found all his 16 strains mannose positive and sorbose negative.

The intermediate strains must indubitably belong to the family *Enterobacteriaceae* and, because of the strongly positive phenylalanine test, to the genus *Proteus*. The only *Proteus* species known to ferment inositol are *P rettgeri* and *P inconstans*. Reactions characteristic of other *Proteus* species (e.g. hydrogen sulphide production, ornithine decarboxylation and rapid gelatin liquefaction) were lacking. The urease reaction must be regarded as positive although for the average strain somewhat slower than for the average *P rettgeri*. It remains to be determined whether the intermediates should be considered *P rettgeri* or urease positive variants of *P inconstans B*

The only biochemical reaction positive for *P inconstans B* (and for the intermediates) in contrast to *P rettgeri* was the fermentation of trehalose. *Brooke* (1) *Ewing et al.* (8) and *Singer & Bar-Chay* (23) found positive trehalose reactions for strains corresponding to *P inconstans B* although the last-mentioned authors also had some trehalose positive *P rettgeri*. *Richard* (19) on the other hand considered trehalose fermentation as one important feature distinguishing *P inconstans B* from *P inconstans A* and *P rettgeri*. Among the reactions usually regarded as characteristic of *P rettgeri*, the intermediates did not exhibit fermentation of mannitol and adonitol. Nor did they ferment erythritol, arabitol, arbutin and salicin or hydrolyse esculin. According to *Kauffmann* (10) the ability to ferment erythritol is a feature unique for *P rettgeri*. This was confirmed by *Namioka & Sakazaki* (16) and *Richard* (19)

The close resemblance between *P rettgeri* and *P inconstans* has been stressed by many authors (3 12, 16, 19 23) If a distinction is to be maintained on the species level it should not be based on urease alone In forming an opinion about intermediate strains all biochemical reactions should be considered. Because of the overall resemblance to *P inconstans B* the strains constituting the intermediate group in the present investigation

should then be regarded as a urease positive variant of this bacterium. The same argument was put forward by *Singer & Bar-Chay* (23).

As the present material represents a rather small area, it cannot be excluded that a majority of the variant strains belong to one clone. It is thus impossible to state anything definite about the incidence in general of the urease positive strains.

Among the strains presented separately in Table 1 one (70141) fulfils all the requirements for a urease negative *P. rettgeri*. Such isolates seem to be very rare in the literature. *Stuart et al.* (25) mentioned a late urease positive *P. rettgeri* which, however, upon subculture, completely lost this property. *Richard* (19) described two strains that he considered to be *P. rettgeri* in spite of negative urease reaction. They were isolated from snake faeces. Urease negative strains of other *Proteus* species were recognized by *Carpenter* (5) and *Edwards & Ewing* (7).

Likewise, the urease positive strain G 82 had most reactions in common with *P. rettgeri* although it did not ferment adonitol, inositol and erythritol.

Strain 103 shared fermentative properties both with *P. inconstans B* and *P. rettgeri*. Particularly noticeable is the lack of urease activity combined with the ability to ferment both trehalose and erythritol. *Edwards & Ewing* (7) do not mention erythritol positive *Providencia* but a small fraction of trehalose positive *P. rettgeri*. On the other hand, *Richard* (19) who apparently does not recognize trehalose positive *P. rettgeri* creates a biogroup of *Providencia* with the characteristics of the present strain. In the opinion of the present author there is no adequate reason for assigning a strain like this to either species. Closer relationship to one of them may be established when further methods of investigation become available.

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Among the strains presented separately in Table 1 one (70141) fulfils all the requirements for a urease negative *P. rettgeri*. Such isolates seem to be very rare in the literature. *Stuart et al.* (25) mentioned a late urease positive *P. rettgeri* which, however upon subculture, completely lost this property. *Richard* (19) described two strains that he considered to be *P. rettgeri* in spite of negative urease reaction. They were isolated from snake faeces. Urease negative strains of other *Proteus* species were recognized by *Carpenter* (5) and *Edwards & Ewing* (7)

Likewise, the urease positive strain G 82 had most reactions in common with *P. rettgeri* although it did not ferment adonitol, inositol and erythritol.

Strain 103 shared fermentative properties both with *P. inconstans B* and *P. rettgeri*. Particularly noticeable is the lack of urease activity combined with the ability to ferment both trehalose and erythritol. *Edwards & Ewing* (7) do not mention erythritol positive *Providencia* but a small fraction of trehalose positive *P. rettgeri*. On the other hand, *Richard* (19) who apparently does not recognize trehalose positive *P. rettgeri* creates a biogroup of *Providencia* with the characteristics of the present strain. In the opinion of the present author there is no adequate reason for assigning a strain like this to either species. Closer relationship to one of them may be established when further methods of investigation become available.

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β-GLUCURONIDASE IN THE STREPTOCOCCAL GROUPS B AND D

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The occurrence of β-glucuronidase was tested in 100 strains of each of the streptococcal groups B and D. Ninety-eight of the group B strains contained the enzyme, whereas no β-glucuronidase was found in any of the group D strains.

β-glucuronidase has been investigated in several kinds of microorganisms (1 6 9 10). In streptococci, the enzyme has been most extensively studied in group A, where a certain difference between different serotypes occurs (5 12, 15). In a material of 50 strains assumed to be enterococci, small amounts were found (4). The enzyme was present in 23 of 42 strains from groups B, C, G and L (5).

The distinction between group B and D colonies is a common problem in clinical bacteriological work. In the present study differences in β-glucuronidase distribution between the two groups and between types within the groups were investigated.

MATERIALS AND METHODS

Bacteria

The following strains have been kindly provided by Dr *R. C. Lancefield** The Rockefeller University New York, U.S.A., and Dr *B. R. Maxted*** Cross-Infection Reference Laboratory Central Public Health Laboratory London, England, through *R H Haug* and by Dr *T W A Little**** Central Veterinary Laboratory Weybridge, England

Group B 090 R 18 RS 21** Pocock**
Group D D 76 N 37** *S. faecalis* gr D**
S. faecium var. *durans* D 5 (96 D)***

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Other group B strains had been isolated as follows 58 from dairy cows with mastitis, 1 from a pig with pericarditis, and 38 from human throat and respiratory passages and female urogenital tract (3)

The remaining group D strains were isolated from human sources: 92 from urine, 2 from wound secretion, 1 from faeces, and 1 from ascites fluid.

Classification

The micro precipitin-technique (8) and Wellcome Streptococcal Grouping Sera (Wellcome Reagents Ltd., Beckenham, England) were used for grouping. The group B strains had been grouped and in most cases been typed earlier (3) using *Lancefield's* precipitin methods (7 8)

The classification of enterococci (see Fig. 1) is based on a review of *Hartman et al.* (2) and a scheme of *Parlова et al.* (11)

The procedures used were as described by *Rogers & Sarles* (13) except for the hydrolysis of gelatine which took place in a medium containing 0.3 per cent beef extract, 0.5 per cent peptone, and 12 per cent gelatine at pH 6.8. The samples were cooled in refrigerator at 4 C for 30 minutes before reading

Tubes were read after incubation for 1 3 and 7 days, and hydrolysis of gelatine in addition after 14 days.

Cultivation

The medium used by *Jacox* (5) was prepared in the following manner: 37 g Brain Heart Infusion (Difco Laboratories, Detroit, Michigan, U.S.A.) was dissolved in 1 l distilled water and autoclaved, whereafter a sterile-filtered buffer solution of 4.0 g glucose, 2.0 g NaCl, 2.0 g $NaHCO_3$, 2.0 g

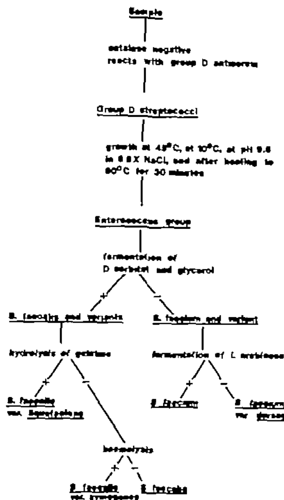


Fig. 1. Scheme for identification of enterococci.

Na_2HPO_4 12 H_2O and 50 ml distilled water was added. Single colonies of all strains were taken from agar plates and inoculated into growth flasks containing 10 ml of the medium. All incubations were carried out aerobically in a NBS Gyrotory Shaker (*New Brunswick Scientific Company, New Brunswick, New Jersey, U.S.A.*) at 37 C for 18 hours.

β-Glucuronidase Analysis

The bacteria were harvested by centrifugation at 10000 × g for 10 minutes, washed in 5 ml sterile phosphate buffer 0.0375 M, pH 6.8 and after centrifugation resuspended in 1 ml of the phosphate buffer.

Streptococcal β-glucuronidase was assayed by *Fishman's* technique (14) as carried out by *Jaco* (5) with minor modifications. About five drops of chloroform were added as preservative and enzyme activator to 1 ml of the bacterial suspensions, which then were pre-incubated for 15 minutes at 37° C. A 0.0015 M solution of phenolphthalein glucuronidase was prepared from the cinchonidine salt (Sigma Chemical Company, St. Louis, Missouri, *U.S.A.*) 0.5 ml was added to the bacterial suspensions which subsequently were incubated at 37 C for 18 hours.

To halt the reaction and to develop colour, 5.0 ml of sterile glycine buffer 0.2 M, pH 10.4, was added.

The suspensions were centrifuged at 10000 × g for 10 minutes, and the optical densities of the supernatants were recorded on a Beckman Model C Colorimeter (Beckman Instruments GMBH, München, Germany) at 540 nm.

A standard curve was made on the basis of commercial bacterial β-glucuronidase and dry phenolphthalein (Sigma Chemical Company) to relate the results to micrograms of phenolphthalein liberated in 1 hour from phenolphthalein β-glucuronide, or "phenolphthalein units" as employed by *Lev[illegible]* & *Conchie* (9)

RESULTS

All but two of the streptococcal group B strains investigated contained β-glucuronidase, whereas no β-glucuronidase was found in any of the group D strains.

One of the two negative group B strains was of bovine, the other of human origin.

TABLE 1. *Distribution of β-glucuronidase Activity*

Group	Type	Number positive	Number negative
B	Total	98	2
	Ia	3	
	Ib	6	
	II	12	
	III	6	
	R	3	1
	X	6	1
	II/R	1	
	Untypeable	28	
	Untyped	53	
D	*Total*	–	100
	S. faecalis	–	31
	S. faecalis var. liquefaciens	–	28
	S. faecalis var. zymogenes	–	34
	S. faecium	–	6
	S. faecium var. durans	–	1

Crossreacted with two typesera.

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BACTERIAL METABOLIZATION OF TAUROLITHOCHOLIC ACID 3-α-SULFATE

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Deconjugation of taurolithocholic acid sulfate is demonstrated in 24 out of 107 strains belonging to genera often found in the intestinal tract of man, whereas bacterial hydrolysis of the sulfate ester bond did not occur

A wide range of intestinal microorganisms are able to transform the steroid nucleus of bile acids or to split the amide bond between the side chain and glycine or taurine (1 3 6, 11 17) The bacterial metabolization of the newly identified lithocholic acid 3-α sulfates (13) has not been investigated.

Lithocholic acid is formed in the intestinal tract of man by bacterial dehydroxylation of chenodeoxycholic acid. It possesses pronounced toxic properties (7) and has been proposed to cause liver disease in man (2, 20) Esterification with sulfuric acid, however may be protective in shortening the biological half life (14) and reducing the toxicity (4) of this compound Desulfation of bile acids by intestinal bacteria therefore might be an important factor in liver damage induced by bile acids

The present study concerns the transformation of taurine conjugated lithocholic acid 3-α-sulfate by intestinal bacteria *in vitro* under anaerobic conditions. The transformation processes studied were hydrolysis of the amide bond and a possible hydrolysis of the sulfate ester

MATERIAL AND METHODS

Bacteriological Procedures

The strains were isolated from duodenal juice, gall-bladder liver small bowel faeces, rectum, urethra, urine, peritoneum and blood. Most of the strains were freshly isolated from the biological material by bacterial routine procedures the remaining strains were formerly isolated at Statens Seruminstitut and stored in extract agar stabs or freeze-dried ampoules.

The microorganisms were grown in a filtered broth medium containing 5 per cent serum, 0.01 per cent glucose, 0.05 per cent haemoglobin and 0.5 per cent yeast extract (Difco) The medium was dispensed in 6 cm high column in 12 × 155 mm glass tubes with cotton wool plugs. To these tubes 0.5 ml sterile bile acid solution was added.

The broth was inoculated with a loopful of organisms from an overnight blood plate culture incubated at 35 C anaerobically in Baird & Tatlock jars. The inoculated medium and an uninoculated control tube were placed in the anaerobic jars and incubated for 4 days at 35 C. At the end of the incubation period, a control of purity and identity was made on the content of all test tubes.

Chemical Procedure

Taurolithocholic acid 3-α-sulfate was synthesized from taurolithocholic acid (Maybridge Research Chemicals, Tintagel, England) as described by *Mumma et al.* (12) The reaction mixture was

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subjected to preparative thin-layer chromatography (ethylacetate/ethanol/ammonia 5.5:1) and subsequently to fractionation on a 4 g Sephadex LH 20 column (18) After washing the gel with 30 ml chloroform/methanol 3 7 containing 0.01 M NaCl, chromatographically pure taurolithocholic acid sulfate could be eluted with 60 ml of the same solvent. The eluate was taken into dryness in a rotatory evaporator and the dry substance was sterilized at 110 C for 30 minutes. A stock solution in saline was made. 0.5 ml of this solution was added to the filtered broth medium to a final concentration of about 1 mg taurolithocholic acid sulfate ester per ml.

After the 4 days of incubation, the bile acids were extracted by means of the resin Amberlite XAD-2 as described by *Makino & Sjövall* (9) The extract was evaporated and redissolved in 0.5 ml methanol and 5 µl was placed on an 0.25 mm thin-layer plate (Kieselgel G, Merck, Darmstadt) which was developed in the butanol-1 system (3) for determination of taurine conjugated and/or sulfated bile acids. Formation of lithocholic acid was tested by gas liquid chromatography One tenth of a sample was dissolved in 1 ml water, pH adjusted to 1 with 2 N HCl, and the water was then extracted with ? ml ether Methyl esters were prepared by reaction with diazomethan Trimethylsilyl ethers were prepared as described by *Makita & Wells* (10) A Packard Instr model 7300/7400 gas chromatograph furnished with a flame ionization detector was used, and the coiled glass columns were packed with 3 per cent SE-30 on Supelcoport mesh 80-100 operation conditions oven temperature 260° C, N flow 60 ml/min. A known standard of lithocholic acid (Supelco Inc.) was run simultaneously

TABLE 1 *The Metabolism of Taurolithocholic Acid Sulfate in 107 Strains. Presence of Taurolithocholic Acid Sulfate (TLS) Lithocholic Acid S lfate (LS) and Taurolithocholic Acid (TL) in the Incubation Medium Tested by Th n Layer Chromatography Presence of Lithocholic Acid (L) Tested by Gas Chromat graphy*

	Number of strains	Positive with respect to			
		TLS	LS	TL	L
Escherichia coli	5	5	0	0	0
Citrobacter freundii	4	4	0	0	0
Enterobacter aerogenes	3	3	0	0	0
Enterobacter cloacae	1	1	0	0	0
Proteus vulgaris	4	4	0	0	0
Proteus m rabilis	3	3	0	0	0
Proteus morganii	2	2	0	0	0
Proteus rettgeri	1	1	0	0	0
Klebsiella pneumoniae	3	3	0	0	0
Kl bsiella oxytoca	2	2	0	0	0
Pseudomonas aeruginosa	2	1	1	0	0
Haemophilus influenzae	1	1	0	0	0
Lactobacillus species	11	11	0	0	0
Coryn bacterium species	1	1	0	0	0
Bacillus species	2	2	0	0	0
Staphylococcus aureus	2	2	0	0	0
St phylococcus albus	3	3	0	0	0
Aerococcus	1	1	0	0	0
Non-haemolytic streptococci	14	14	0	0	0
Streptococcus faecalis	2	1	1	0	0
Bacteroides species	14	7	7	0	0
Veillonella species	2	2	0	0	0
Anaerobic *Lactobacillus* species	4	4	0	0	0
Anaerobic *Corynebacterium* species	2	2	0	0	0
Clostridium wel hii	15	0	15	0	0
Anaerobic gram-positive cocci growing in clusters	3	3	0	0	0
Total	107	83	24	0	0

RESULTS

107 strains were tested (Table 1). Taurolithocholic acid sulfate was deconjugated by *Clostridium welchii*, *Bacteroides* species, *Streptococcus faecalis* and *Pseudomonas aeruginosa*. When deconjugation occurred, it was always complete as judged by thin-layer chromatography. Transformation of taurolithocholic acid sulfate to taurolithocholic acid or lithocholic acid could not be demonstrated.

DISCUSSION

Palmer & *Bolt* (15) showed that the major part of lithocholic acid excreted in the bile is sulfated. Due to the pronounced pathological significance of lithocholic acid (7) and the profoundly altered physiological properties of its sulfate ester (4, 8, 14, 19) it is of interest to know how bile acid sulfates are metabolized by the intestinal microorganisms, and especially whether or not the ester link resists bacterial activity. The distribution of steroid sulfatase seems to be very sporadic in nature: it occurs in many molluscs and mammals but is apparently absent in other animals and protists. The search for the enzyme, however, has not been exhaustive (16). In the present work we have screened a variety of bacteria which are representative of human intestinal flora, but a bacterial bile acid sulfatase could not be demonstrated. The extent to which sulfated bile acids are deconjugated seems to be the same as that of non-sulfated acids (17).

A bacterial bile acid sulfatase may, of course, be present in microorganisms other than those tested here, or it may require special conditions found in the intestinal tract of man. This study provides no evidence for the existence of such a sulfatase and does not support the theory that bacterial colonization of the small bowel may cause an abnormal production and absorption of lithocholic acid due to hydrolysis of its sulfate ester leading to hepatic disease in man.

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EPIDEMIOLOGICAL INVESTIGATIONS OF THE RESPIRATORY TRACT BACTERIOLOGY IN PATIENTS WITH CYSTIC FIBROSIS

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Seventy patients with cystic fibrosis treated as out-patients in a cystic fibrosis clinic have been followed during one year by monthly bacteriological examinations of tracheal secretions. The daily impression obtained in the laboratory is expressed by the mean point prevalence rate of *Pseudomonas aeruginosa*, 44 per cent, *Staphylococcus aureus* 39 per cent, *Haemophilus influenzae* 17 per cent, *Diplococcus pneumoniae* 8 per cent, and miscellaneous other bacteria, mainly Enterobacteriaceae 8 per cent. The fluctuations of the bacteriology are described by additional epidemiological terms. At one or more examinations during the study (period prevalence rate) 90 per cent of the patients harboured *St. aureus* 64 per cent *Ps. aeruginosa* (mainly mucoid strains) 64 per cent *H. influenzae* 37 per cent *D. pneumoniae* and 30 per cent miscellaneous other bacteria, mainly Enterobacteriaceae. This pattern was found in all age groups with minor age-dependent modifications, especially as regards Enterobacteriaceae. *Ps. aeruginosa* was predominating as regards chronic colonization which reflects the most difficult therapeutic problems, the period prevalence rate being 39 per cent in contrast to *St. aureus* 10 per cent, and *H. influenzae* 1 per cent. *St. aureus* was predominating as regards new colonization and recolonization which reflect the problems of prevention, the incidence rate of new colonization and recolonization per risk group being 84 per cent, followed by *H. influenzae* 62 per cent, *Ps. aeruginosa* 43 per cent, and *D. pneumoniae* 30 per cent. The results show that the bacteriological problems in cystic fibrosis are still considerable as regards therapy as well as prevention. Although many species may colonize the respiratory tracts of these patients, the main clinical problems concern *Ps. aeruginosa* and *St. aureus* the reason why is discussed.

Patients (pts.) suffering from cystic fibrosis (CF) are known to contract frequent respiratory tract infections (6, 12, 18, 29 30). Despite the increasing number of different chemotherapeutic drugs available to combat these infections, chronic respiratory tract infections with progressing respiratory failure is still considered the main cause of death in CF (4 12, 23) although the survival time of CF pts. has improved substantially during the present two decades (23)

The bacteriology of CF has been the subject of many reports (1 4 6, 12, 18 22, 23

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29 30) which generally agree in finding *Staphylococcus aureus* (*St aureus*) *Pseudomonas aeruginosa* (*Ps aeruginosa*) *Haemophilus influenzae* (*H influenzae*) and *Diplococcus pneumoniae* (*D pneumoniae*) to be the species most frequently associated with CF Especially *St aureus* and *Ps aeruginosa*, often found together in the respiratory tracts of these pts., have been a characteristic finding in contrast to findings in other groups of pts. with chronic respiratory tract infections where *D pneumoniae* and *H influenzae* are the pathogens most often isolated (12, 18 28)

The aim of the present study was to investigate the epidemiology of the bacterial colonization in the respiratory tracts of CF pts. currently treated and followed as out pts. in a CF centre. The therapeutic results will not be subject to evaluation in this study

MATERIALS AND METHODS

Patients

About 90 CF pts. are followed in the Pediatric Clinic TG Rigshospitalet Tagensvej the centre of CF treatment in Denmark. Seventy of these pts. were included in the study as they were the only ones who complied with the following demands All pts. should have been followed as out-pts. for at least one year in the clinic (mean 3.3 years, range 1–16.5 years) where they were examined every or every other month If necessary they were hospitalized in the clinic. Sex and age distribution of the pts 42 males (mean age 9 6 years, range 2–22 years) and 28 females (mean age 8.8 years, range 1–20 years) The diagnostic criteria have been reported previously (14)

Period of this Study

July 1st, 1972 to June 30 1973 In this period, the mean number of pts. examined each month was 63 (range 55–70) each pt. being subjected to, on an average, 10 8 monthly examinations during the year of this study (range 7–12)

Treatment

The pts. are subject to daily extensive and differentiated treatment at home, including sleeping in mist tents, inhalation therapy with mucolytic agents and lung physiotherapy Concerning the antibiotic therapy from the day of diagnosis, all pts. receive permanently oral sulphonamide unless this drug is not tolerated. Sixty-six of the 70 pts. in this study received sulphonamide. If indicated by clinical and bacteriological examinations, specific antimicrobial therapy is given in accordance with the results of sensitivity testing of the bacteria isolated. In this clinic, bacteriological indication for antimicrobial therapy includes that the bacteria in question have been cultivated as well as found by microscopy according to the principles described below

Bacteriological Examinations

Sputum or tracheal secretion obtained by aspiration through a catheter and collected in sterile containers are brought to the laboratory in cooling boxes and examined immediately The most purulent part of the material is exposed to the following examinations 1) gram stained films are examined by microscopy by a bacteriologist and the flora associated with areas consisting of respiratory epithelial cells and pus cells but without squamous epithelial cells is described, whereas the flora associated with oral epithelial cells is considered to be predominantly of oral or pharyngeal origin, and not described (21) 2) Inoculation of 4 pieces, the size of half a pea, of the material on 2 blood agar plates and 1 differential medium plate for gram negative rods One of the blood agar plates is used for primary testing of antibiotic sensitivity (Neo-sensitabs® Rosco A/S, Taastrup, Denmark) the other blood agar plate is furthermore inoculated with a streak of *St aureus* to diagnose *H influenzae* by means of the satellite phenomenon The plates were incubated for 20 h at 35 C. The cultivated bacteria were identified using standard methods (2)

Media for Cultivating the Bacteria

The media used were standard media from Statens Seruminstitut

Blood agar plate 4 g Na_2HPO_4, $12H_2O$, 5 g NZAmin type b (Sheffield) 2 5 g Orthana yeast extract (Orthana) 1 66 g NaCl, 0.03 g soap, 10 5 g glucose, 50 ml horse blood, 9 g Bacto-Agar (Difco) 1 litre ion-exchanged tap water pH 7.3

Differential medium 10 g peptone (Orthana) 5 g yeast extract (Oxoid L 21) 5 g NaCl, 1 ml Maranil 5 per cent, 2 ml $Na_2S_2O_3$ 50 per cent, 10 ml Bromthymolblue 1 per cent, 27 ml lactose 33 per cent, 1.2 ml glucose 33 per cent, 10 g Bacto-Agar (Difco) 1 litre ion-exchanged tap water, pH 7 7–7.8.

Epidemiological Terms Used (24)

Point prevalence (*point prevalence rate* (*PoPR*)) be at is the number of pts (percentage of pts.) who at a designated time of the investigation (at the monthly examination) were harbouring the bacteria in question in the respiratory tract. The mean value of the 12 monthly PoPR is designated *mean point prevalence rate* (*mPoPR*)

TABLE 1 *Results of the Bacteriological Findings During 1 Year of Monthly Examinations of Tracheal Secretions or Sputum from 70 Patients with Cystic Fibrosis*

	Period prevalence (1 year)		Mean point prevalence (mean $\bar{x}$, and range r of monthly examinations during 1 year)		Incidence of new colonization (1 year)		Incidence of recolonization (1 year)	
	total	chronical	total	chronical	total	chronical	total	chronical
Pseudomonas aeruginosa	45 (64%)	27 (39%)	$\bar{x}$ 31 (44%) r: 27–39	$\bar{x}$ 25 (36%) r: 20–32	10 (14%)	6 (8.6%)	8 (11%)	1 (1.6%)
Staphylococcus aureus	63 (90%)	7 (10%)	$\bar{x}$ 27 (39%) 15–37	$\bar{x}$ 4 (6.2%) r 0–13	6 (8.6%)	4 (5.7%)	25 (37%)	0
Haemophilus influenzae	45 (64%)	1 (1.4%)	$\bar{x}$ 12 (17%) r: 7–16	$\bar{x}$: 1 (1.6%) r 1	10 (14%)	0	29 (41%)	0
Diplococcus pneumoniae	26 (37%)	0	$\bar{x}$ 5 (7.8%) r 1–11	$\bar{x}$ 0	8 (11%)	0	10 (14%)	0
Miscellaneous								
Escherichia coli	12 (17%)	0	$\bar{x}$ 2.4 (3.8%)	0	Not calculated		Not calculated	
Klebsiella species	6 (8.6%)	0	$\bar{x}$ 0.8 (1.3%)	0	Not calculated		Not calculated	
Proteus species	3 (4.3%)	0	$\bar{x}$ 0.8 (1.3%)	0	Not calculated		Not calculated	
Group A Streptococci	1 (1.4%)	0	$\bar{x}$ 0.1	0	Not calculated		Not calculated	
B. [illegible]	1 (1.4%)	0	$\bar{x}$ 0.1	0	Not calculated		Not calculated	
Atypical Mycobacterium	1 (1.4%)	1 (1.4%)	$\bar{x}$ 0.8 (1.3%)	$\bar{x}$ 0.8 (1.3%)	Not calculated		Not calculated	

Occurrence of the 4 predominating bacteria and miscellaneous other bacteria in the respiratory tracts of 70 cystic fibrosis patients. The results are expressed in epidemiological terms (see materials and methods)

Period prevalence (period prevalence rate (P PR)) is the number of pts. (percentage of pts.) who during the one-year period of this study have harboured the bacteria in question in the respiratory tract at one or more examinations.

Incidence of new colonization (incidence rate of new colonization (IRN)) is the number of pts. (percentage of pts.) who during the one-year period of the study have harboured the bacteria in question in the respiratory tract for the first time (to our best knowledge).

Incidence of recolonization (incidence rate of recolonization (IRR)) is the number of pts. (percentage of pts.) who at the first examination during the one-year period of this study did not harbour the bacteria in question, although they had harboured it previously but who during the one-year period of this study harboured the bacteria again in the respiratory tract at one or more examinations. (It should be stressed that this term describes the number of *pts.* in whom recolonization occurred, i.e. only one period of recoloniza-

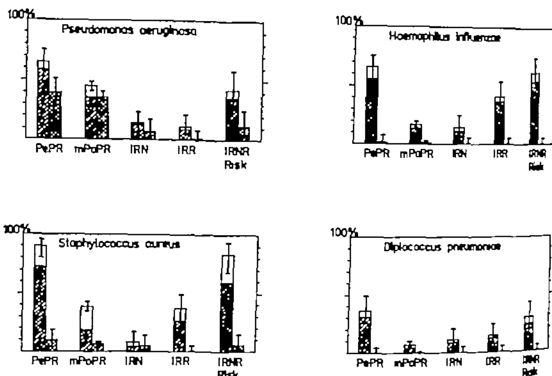


Fig 1 One year period prevalence rate (*PePR*) mean point prevalence rate (*mPoPR*) incidence rate of new colonization (*IRN*) incidence rate of recolonization (*IRR*) and incidence rate of new colonization + recolonization per risk group (*IRNR Risk*) of the 4 predominating bacteria in the respiratory tracts of 70 cystic fibrosis patients. Each rate is given by 2 columns the first column represents the percentage of the patients who had harboured the bacteria according to the definitions of the rates, the second double-hatched column represents the percentage of the patients who had harboured the bacteria chronically according to the definitions of the rates. The vertical bars represent the 95 per cent confidence limits of the rates. The hatched part of the first columns represents the results of the simultaneous microscopic examinations of gram stained films (see Materials and Methods where explanations of the terms used are also given)

tion per pt. is included though more than one episode of recolonization occurred in some of the pts. during the one-year study)

Incidence rate of new colonization and recolonization per risk group (IRNR Risk) is the sum of incidence of new colonization and recolonization in percentage of the number of pts. who did not harbour the bacteria in question in the respiratory tract at the first examination during the one year period of this study. The relation between the "rates" can be expressed by the equation PoPR at the beginning of the study (July 72) + IRN + IRR = PePR.

Chronically colonized pts Pts. are considered chronically colonized with the bacteria in question if they have harboured the bacteria in the respiratory tract at every bacteriological examination for at least 6 months.

Statistical Methods (3)

The χ^2-test with Yates correction if indicated.

RESULTS

In Table 1 is given the results of the one-year bacteriological findings in the sputum or tracheal secretions of the 70 CF pts. It is seen that *Ps aeruginosa*, *St. aureus* and *H influenzae* are the species most frequently isolated from these pts., followed by *D pneumoniae* and miscellaneous other bacteria, mainly Enterobacteriaceae.

In Fig 1 the data from Table 1 is given as percentage of the pts. or risk groups of pts. in this study (rates) The results of microscopic examinations of gram stained films are also given, i.e. the percentage of pts. in whom the microscopic findings agree with the results of cultivating the sputum or tracheal

secretions, according to the principles described in Materials and Methods.

It is seen from Table 1 and Fig 1 that most of the pts. have harboured *St aureus* during the 1-year period (period prevalence rate). *Ps. aeruginosa* as well as *H influenzae* have been isolated from 2/3 of the pts., whereas *D pneumoniae* was isolated from slightly more than 1/3 of the pts. The differences between these frequencies are significant ($p<0.0005$). Miscellaneous other bacteria were more seldom isolated, and even the Enterobacteriaceae family was not as frequent as any of the 4 predominating species with respect to *period prevalence rate* as well as *mean point prevalence rate*. Sixty-nine per cent (95 per cent confidence limits 53 per cent–82 per cent) of the pts. who harboured *Ps. aeruginosa* at one or more examinations during the period of the study presented mucoid strains.

With respect to *period prevalence rate of chronic colonization Ps aeruginosa* is by far the predominating organisms, whereas only few of the pts. harboured *St aureus* chronically and only one pt. harboured *H influenzae* chronically and none harboured *D pneumoniae* chronically. One pt. harboured atypical Mycobacteria chronically whereas no other member of the miscellaneous group caused chronic colonization. (Table 1 and Fig. 1). These frequencies differ significantly from each other ($p<0.0005$). With respect to *chronic colonization* the *mean point prevalence rates* are close to the *period prevalence rates* as regards these species. Also here the differences are significant ($p<0.0005$).

The *incidence rate of new colonization* show no significant differences between the 4 main species (Table 1 and Fig. 1). If the *incidence rate of new chronic colonization* is considered, only *Ps. aeruginosa* and *St aureus* (and the Mycobacterium) are represented, in contrast to *H influenzae* and *D pneumoniae* here the differences are significant ($p<0.01$).

The *incidence rate of recolonization* shows that *H influenzae* and *St. aureus* are predominating whereas *Ps. aeruginosa* and *D pneumoniae* are less frequent (Table 1 and Fig 1) these differences are significant ($p<0.0005$). However as can be seen from Fig. 1 the different percentages of the pts. who harbour the different bacteria chronically (*period prevalence rate and mean point prevalence rate of chronic colonization*) indicate that the *rates incidence rate of new colonization* and *incidence rate of recolonization* are influenced by the number of pts. who are chronically colonized with the bacteria in question. To corrigate for this, calculations have been made to determine the *incidence rate of new colonization and recolonization per risk group (IRNR Risk)*. The results (Fig 1) show that *St.aureus* is the predominating organism (84 per cent) followed by *H influenzae* (62 per cent) *Ps aeruginosa* (43 per cent) and *D pneumoniae* (30 per cent). These differences are significant ($p<0.0005$). With respect to *chronic colonization*, the *incidence rate of new colonization and recolonization per risk group* shows that *Ps aeruginosa* is the predominating organism (12 per cent) followed by *St aureus* (6 per cent) whereas other species (except the Mycobacterium) were not found. These differences are significant ($p<0.005$).

Fig 2 shows the *point prevalence rates* for each of the 12 months. No striking seasonal differences are seen, but *St aureus* tend to be more frequent during and after the summer holliday and *D pneumoniae* tend to be more frequent during the autumn and the winter. If the 4 predominating species and the miscellaneous group are combined and *point prevalence rates* and *mean point prevalence rate* for harbouring one or more of the species are calculated, it appears from Fig. 2 that, on an average, 81 per cent of the patients (95 per cent confidence limits 78 per cent–84 per cent) were harbouring some of these bacteria in the respiratory tract with no seasonal variation. As regards *chronic colonization* similar calculations show that an average of 41 per cent (95 per cent confidence limits 36 per cent–45 per cent) of the pts. harbour some of these bacteria chronically. If these calculations are made

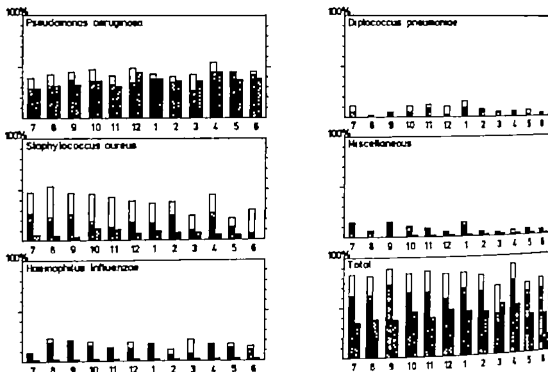


Fig 2 Results of monthly bacteriological examinations of tracheal secretions or sputum from 70 cystic fibrosis patients. The results are expressed as point prevalence rates of the monthly examinations. Each month is given by its number. The results are given for each of the 4 predominating bacteria, miscellaneous other bacteria, and the total of these groups. For explanation of the columns see text to Fig 1. The miscellaneous group (see Table 1) consists mainly of Enterobacteriaceae, other species from the miscellaneous group are indicated by the bl ck part of the columns. The only chronic colonization by this group was caused by an atypical Mycobacterium.

with respect to *period prevalence rates* 100 per cent (95 per cent confidence limits 93 per cent–100 per cent) of the pts. harboured some of these bacteria in the respiratory tract, 44 per cent even chronically (95 per cent confidence limits 32 per cent–57 per cent).

Fig 3 shows the *period prevalence rates* of simultaneous isolation of 2 or more species from the same pt. It is seen that the combinations of *Ps aeruginosa* with *St aureus* (46 per cent) and *St aureus* with *H influenzae* (31 per cent) are the most frequent findings whereas other combinations are found in 10 per cent or less of the pts. Members of the miscellaneous group were found in combination with 1 or more of the 4 predominating species in 0–4 per cent of the pts.

If pts. chronically colonized with *Ps aeruginosa* and pts. *not* chronically colonized with *Ps aeruginosa* are compared as regards *period prevalence rates* of other species, it appears that *St aureus* is isolated with the same frequency in the 2 groups, whereas *H influenzae* is isolated significantly more frequently from pts. not chronically colonized with *Ps. aeruginosa* (88 per cent) than from pts. chronically colonized with *Ps aeruginosa* (26 per cent) ($p<0.0005$). The same tendency is found with respect to *D pneumoniae* (51 per cent–15 per cent, $p<0.0025$) and the miscellaneous group (40 per cent–15 per cent, $p<0.05$).

If similar calculations are performed with regard to pts. chronically harbouring *St. aureus* and pts. *not* chronically harbouring *St aureus* it appears that *H influenzae* is isolated more frequently from pts. not harbouring *St aureus* chronically (70 per cent) than

the youngest pts. (Fig 4) The importance of most of the bacteria (Enterobacteriaceae) in the last group is uncertain, although they have been incriminated as "indirect pathogens" being able to produce penicillinase (25)

The reason for the varying differences between *mean point prevalence rate* and *period prevalence rate* in the different species are found in differences in new colonization and especially *recolonization*. *Incidence rates of recolonization* are by far the highest for *St aureus* and *H influenzae* somewhat lower for *D pneumoniae* and lowest for *Ps aeruginosa*. The reason for this is that infections with *St aureus H influenzae* and *D pneumoniae* are treatable by means of antibiotics moreover colonization, especially with the 2 last mentioned species, is frequently discontinued without specific treatment (4 21 23 28) and thus, colonization with these species will be recurrent rather than chronic predominantly different pts. will therefore harbour these species at different times of the investigation and this will mainly be reflected in the *period prevalence rate* less in the *mean point prevalence rate* The opposite applies to *Ps aeruginosa* where discontinuance of colonization is more seldom and the therapeutic efforts generally are disappointing (4 11 16, 21) thus, in contrast to the other species, it will mainly be the same pts. who will be harbouring *Ps aeruginosa* at different times of investigation. The *incidence rate of recolonization* is proportionally low and this is reflected in the rather small difference between *period prevalence rate and mean point prevalence rate* the difference mainly being accounted for by *incidence rate of new colonization*

The best measure of the frequency at which CF pts. are colonized by the 4 predominating species (reflecting the problems of prevention) is probably the *incidence rate of new colonization and recolonization per risk group* showing that *St aureus* and *H influenzae* are predominating in this respect, whereas *Ps aeruginosa* and *D pneumoniae* are less frequent.

The number of pts. chronically colonized with the different bacteria reflects the inefficiency of the therapeutic efforts. It is seen that, in this respect, *Ps. aeruginosa* represent the predominating problem as reported elsewhere (4 11 23 28) moreover *Ps. aeruginosa* isolated from this group of pts. are often mucoid strains, in this material nearly 70 per cent, which is in accordance with other reports (1 4 6 14 18, 23) As therapeutic efforts are mainly disappointing in cases of *Ps aeruginosa*, the efforts of prevention are especially important, but seem to be largely ineffective according to the results of this study

It appears from Fig 3 that nearly all combinations of the 4 predominating species isolated simultaneously were found. However, in pts. chronically harbouring either *Ps. aeruginosa* or *St aureus H influenzae* and in the former case also *D pneumoniae* and the miscellaneous group were found less frequently than in pts. not harbouring *Ps. aeruginosa* or *St aureus* chronically As long as selective media for these species are not used (28) the significance of these findings is uncertain, although similar results have been reported by others (1 4 22)

Nearly all the pts. in this study were on long term sulphonamide prophylaxis. The value of this is doubtful as can be seen from the figures, and sulphonamide prophylaxis has therefore now been abandoned.

It is a general impression from the literature (1 4 6, 18 22 23 28, 29 30) and from this clinic (11 21) that *St. aureus* and mucoid strains of *Ps aeruginosa* are the real pathogens in the respiratory tract of CF pts. Other species may infrequently cause clinical sign of infection and infrequently need chemotherapy in this clinic, although the results show that they frequently colonize the respiratory tract of these pts. Moreover the humoral immune response in CF pts is very pronounced, antibodies against bacteria, especially *Ps aeruginosa* and *St aureus* are very frequent and high titred in these pts. (1 4, 7 9 10 17 19 23 27 29 30, 31) and CF pts. very seldom, if ever contract infection out-

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PSEUDOMONAS AERUGINOSA INFECTION IN CYSTIC FIBROSIS

Relationship between mucoid strains of Pseudomonas aeruginosa *and the humoral immune response*

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The occurrence of *Pseudomonas aeruginosa* in the respiratory tract of 70 cystic fibrosis patients and the occurrence of precipitins against *Ps. aeruginosa* in sera from the same patients has been investigated during one year by means of monthly bacteriological examinations of tracheal secretions and by means of crossed immunoelectrophoresis of polyvalent *Ps. aeruginosa* antigen against sera from the patients. The one-year period prevalence rate of patients harbouring *Ps. aeruginosa* was 64 per cent. In newly colonized patients and in intermittently colonized patients non-mucoid strains were predominating, whereas mucoid strains were predominating in chronically colonized patients. The occurrence of mucoid strains, especially in chronically colonized patients, was associated with a significantly higher number of precipitins against *Ps. aeruginosa* than the occurrence of non-mucoid strains. Males chronically colonized with mucoid strains presented a significantly higher number of precipitins than females chronically colonized with mucoid strains and the number of precipitins was correlated with the duration of the chronic colonization with *Ps. aeruginosa* in males in contrast to females. The results are in accordance with the hypothesis that mucoid substance could be a virulence factor because it might inhibit the opsonizing effect of the precipitins on the mucoid cells and the complement dependent lysis of the cells thereby favouring mucoid strains at the expense of non-mucoid strains in the respiratory tract of cystic fibrosis patients.

Pseudomonas aeruginosa (*Ps. aeruginosa*) are among the bacteria most frequently isolated from the respiratory tracts of patients (pts.) suffering from cystic fibrosis (CF) (8, 13, 16, 17). *Ps. aeruginosa* isolated from such pts. are very often mucoid strains (1, 4, 6–11, 13, 17). The production of mucoid substance has been shown to be phage-dependent (18) and moreover it has been shown that this phage, and thus the mucoid substance, is very easily lost (3, 14, 18, 20). In most other groups of pts. such strains are seldom isolated (1, 2, 7–9, 15).

Høiby & Axelsen (1973) have recently suggested that the selective factor favouring the mucoid strain in CF is the immune response, as many and high titred antibodies against *Ps. aeruginosa* were found in these pts. (1, 2, 4, 10, 11). The mucoid substance might fav

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our the colonization with strains producing this substance at the expense of non-mucoid strains by inhibiting the opsonizing effect of antibodies on the bacterial cells and the complement dependent lysis of the cells.

The aim of this study was to further investigate the relationship between *Ps. aeruginosa* especially the mucoid strains, and the humoral immune response. For this purpose, the respiratory tract bacteriology of 70 CF pts. has been followed during one year and sera from these pts. have been examined by means of crossed immunoelectrophoresis for the occurrence and number of precipitating antibodies against *Ps aeruginosa* considering the correlation between the number of precipitins and the titres of the strongest precipitins (11 15) The results of the bacteriological examinations with respect to the occurrence of *Ps aeruginosa* and other bacteria are published in another article (13) the results with respect to the proportion between mucoid and non mucoid strains of *Ps aeruginosa* and the results of the serological investigations are reported in the present work.

MATERIALS AND METHODS

Patients

Seventy CF pts. were included in the study All pts have been followed as out-pts. for at least one year by bacteriological examinations of the tracheal secretions or sputum every or every other month in the Pediatric Clinic TG Rigshospitalet Tagensvej, Copenhagen. Details of the age and sex distribution, and the period of control in the clinic have been reported elsewhere (13) the same applies to the diagnostic criteria (11) and details about the one-year period of the study (13)

Bacteriological Examinations

Sputum or tracheal secretion obtained by aspiration trough a catheter was investigated by microscopy of gram stained films and by cultivation on 2 petri dishes with 5 per cent blood agar and 1 petri dish with differential medium for gram negative rods. The details concerning the bacteriological examinations are reported elsewhere (13)

Crossed Immunoelectrophoresis

Sera from the patients were investigated for the occurrence and number of precipitins against *Ps. aeruginosa* by means of a polyvalent standard antigen (St Ag) consisting of water-soluble constituents obtained from 4 different O-groups of *Ps. aeruginosa* by sonication as described previously (11). The crossed immunoelectrophoreses of St-Ag against pts. sera were carried out in microtechnique (on 5 × 5 cm glass plates) as described by Weeke (1973) using 1 per cent agarose gel (thickness of gel 1 mm) in barbital buffer pH 8.6, ionic strength 0.02 in the gel and the wicks and 0.05 in the buffer vessels. The first dimension electrophoreses of St-Ag (? µl) were run at 12° C applying 10 V per cm for 30 min. The second dimension electrophoreses were run at 12° C applying 1.3 V per cm for 20 h The second dimension gel contained 15 µl of pts. serum per cm

After the second dimension electrophoresis the gel was washed, dried and stained with Coomassie brilliant blue as described previously (11) Sera from the pts. were stored at −30° C with NaN_3 added to a concentration of 15 mM. Several sera from each pt were investigated during the period, and the highest number of precipitins demonstrated in each pt. are reported in this work. Results obtained by a comparison of findings at examinations of subsequent sera from the pts. are to be published later

Epidemiological terms are used as described elsewhere (13) *Period prevalence (period prevalence rate)* is the number of pts. (percentage of pts) who during the one-year period of this study have harboured *P aeruginosa* in the respiratory tract at one or more examinations. Pts. included in this group are designated *CF + P* if the strain was mucoid *CF + MP* if the strain was non-mucoid *CF + NMP* and if the strain was alternating non-mucoid and mucoid at different times of examination *CF + NMP/MP* If the pts. had harboured *Ps. aeruginosa* in the respiratory tract at every bacteriological examination for at least 6 months, they are considered *chronically colonized* and designated *CF + P(c)* If not, they are designated *intermittently colonized* (*CF + P(i)*). *CF — P* are pts who during the one-year period of this study have not harboured *Ps. aeruginosa* in the respiratory tract.

Incidence of new colonization (incidence rate of new colonization) is the number of pts (percentage of pts.) who during the one-year period of the study harboured *Ps. aeruginosa* in the respiratory tract for the first time (to the best of our knowledge)

Chemicals

Coomassie brilliant blue Michrome no 1137 was obtained from E Gurr Ltd., London, England. Agarose for the electrophoreses was received from L'Industrie Biologique Francaise S.A., (Indubiose A 37 Batch FJ 4354) All the electrophoreses were run using the equipment supplied by Dansk Laboratoriesudstyr A/S, Copenhagen, Denmark

TABLE 2. *Occurrence of Mucoid and Non-mucoid Strains in Cystic Fibrosis Patients Colonized Intermittently or Chronically with* Pseudomonas aeruginosa

	Total	No. of CF + NMP	No. of CF + NMP/MP	No. of CF + MP
No. of CF + P(c)	27	1 (4%)	5 (18%)	21 (78%)
No. of CF + P(i)	18	13 (72%)	5 (28%)	0

For explanation of abbreviations used see Materials and Methods.

the crossed immunoelectrophoreses are shown in Fig. 1 and 2. Thirty-one (44 per cent) pts. had precipitins against *Ps. aeruginosa* and 29 of these harboured *Ps. aeruginosa* chronically or intermittently; one pt. had previously harboured *Ps. aeruginosa* although he did not harbour this species during the period of this study. As far as we are informed, the last pt. had never harboured *Ps. aeruginosa*.

There is a highly significant difference between the presence of precipitins in the group of pts. intermittently or chronically harbouring *Ps. aeruginosa* and the group of pts. who had never harboured *Ps. aeruginosa* ($p<0.0005$). This difference is, however, due to the group of chronic colonized pts., as there is no significant difference between the presence of precipitins in pts. never colonized and pts. intermittently colonized with *Ps. aeruginosa*.

Moreover it seems (Table 1) that the occurrence of precipitins and the number of precipitins per serum are *lowest* in pts. intermittently colonized with non-mucoid *Ps. aeruginosa* (Fig. 1); it is *intermediate* in pts. intermittently colonized with alternating non-mucoid and mucoid strains or chronically colonized with non-mucoid or alternating non-mucoid and mucoid strains, and *highest* in pts. chronically colonized with mucoid strains of *Ps. aeruginosa* (Fig. 2). The number of precipitins per serum in pts. chronically colonized with mucoid strains is significantly higher than the number of precipitins per serum in pts. chronically colonized with non-mucoid or alternating non-mucoid and mucoid strains ($p<0.01$).

During the period of the study 10 of the pts. contracted *Ps. aeruginosa* in the respiratory tract for the first time (incidence rate of new colonization = 14 per cent) (Table 3). Eight (80 per cent) of these pts. harboured non-mucoid strains at the time of the first isolation and two were colonized with mucoid strains (20 per cent). In Table 3 it is seen that, at the end of the study, three of these pts. had become chronically colonized, two of these with mucoid strains. These two pts., 1½ year and 2¼ years old, now produced many precipitins, whereas the pt. chronically colonized with alternating non-mucoid

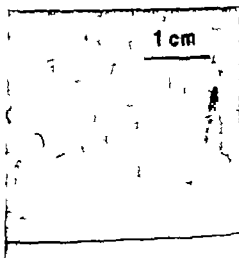


Fig. 1. Crossed immunoelectrophoresis of 2 µl St. 4 against patient serum (15 µl/cm²) in the second dimension gel. One very weak precipitate could be seen in this plate. This was the only serum presenting *Ps. aeruginosa* precipitins from a patient belonging to the group intermittently colonized with non-mucoid *Ps. aeruginosa*. (1. dimension electrophoresis: anode to the right. 2. dimension electrophoresis: anode at the top. Staining: Coomassie brilliant blue.)

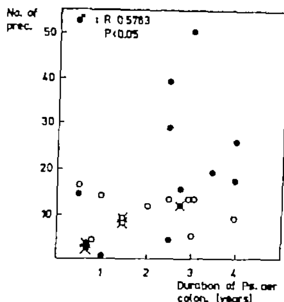


Fig. 3. Relationship between the number of *Ps. aeruginosa* precipitins (No. of prec.) and the duration of the chronic *Ps. aeruginosa* colonization (Ps. aer. colon.) in 24 cystic fibrosis patients chronically colonized with these bacteria. (Males ● and females ○ harbouring mucoid *Ps. aeruginosa*. Males ⌘ and females ⌗ harbouring non-mucoid strains or alternating harbouring mucoid or non-mucoid strains of *Ps. aeruginosa*). Spearmans correlation coefficient R and the corresponding p value are given for the males.

tasis and other chronic suppurations such as pyonephrosis, the situation is similar to CF: mucoid strains are frequent, and so are precipitins against *Ps. aeruginosa* (1, 2, 15).

It appears from Tables 1–3 that non-mucoid strains predominate in the newly colonized and intermittently colonized CF pts., whereas mucoid strains predominate in the chronically colonized pts. From these data, and in accordance with the findings obtained by *Doggett & Harrison* (1966, 1969a & b) it seems that CF pts. initially become colonized with predominantly non mucoid strains, but as the colonization goes on and becomes chronic, the mucoid strains will gradually be predominating.

It is also seen from Tables 1 & 3 and from Figs. 1–3 that mucoid strains are associated with a strong and differentiated humoral immune response, considering the positive correlation between the number of precipitins and the titres of the strongest precipitins (11, 15), whereas non-mucoid strains are associated with significantly fewer precipitins; this is in accordance with previous findings (1, 2, 4, 9–11).

The observed difference between males and females colonized chronically with *Ps. aeruginosa* as regards the number of precipitins is also reflected in Fig. 3 from which it appears that females do not reach the highest numbers of precipitins. Any explanation of this difference cannot be offered at the present time.

It has previously been shown that increased numbers of circulating neutrophil granulocytes and lymphocytes (bone marrow-derived as well as thymus-derived) are found in CF pts. chronically colonized with mucoid strains of *Ps. aeruginosa* and presenting many precipitins against this bacteria (14). Moreover, *CF pts. harbouring these bacteria* demonstrate significantly changed serum proteins in proportion to normal controls, especially as regards the so-called "acute phase proteins" (12), suggesting active tissue damage. It has also been shown that the number of *Ps. aeruginosa* precipitins are positively correlated with the concentration of some of the "acute phase" proteins, notably haptoglobin which is especially well-correlated with lung disease (12).

It appears from the data of the present *study and from the literature* cited that mucoid strains of *Ps. aeruginosa* are associated with 1) chronic colonization, 2) a strong and differentiated humoral immune response with coincident alterations in lymphocytes, neutrophil granulocytes and acute phase proteins suggesting active tissue damage in the lungs. *These evidences* support the impression that mucoid strains of *Ps. aeruginosa* are real pathogens in *CF pts.* (1, 2, 8, 10, 11) and furthermore suggest that mucoid strains are more virulent than non-mucoid strains which is in accordance with other reports (2, 4, 8, 10, 19). As no difference between *mucoid strains* and non-mucoid strains other than the mucoid substance has been found, this *substance* might be con-

sidered a virulence factor in the respiratory tracts of CF pts. (4 8 10 19)

Production of mucoid substance has been shown to be phage dependent (18) and also to be a property easily lost during subculture, in other words, maintenance of mucoid strains requires regular selection of colonies (3 15 18, 20) It has therefore been proposed that some selective factor must be active in CF pts. in order to favour mucoid strains (4 8, 11 18, 19) However such a selective factor must also be active in pts. with bronchiectasis and other chronic suppurations where the situation is similar to CF mucoid strains of *Ps. aeruginosa* are frequent and these pts. produce many precipitins a gainst this bacteria (1 2, 15) On the other hand, this selective factor seems to be weak or absent in other groups of pts., for example in pts. with chronic bronchitis or pts. with post operative wound infections, where non-mucoid strains of *Ps. aeruginosa* are predominating and precipitins are infrequent findings (1 2, 15)

Hoiby & *Axelsen* (1973) have therefore proposed that the selective factor working in CF pts. as well as in pts. with other chronic suppurations is the immune response of the pts. The great amount of mucoid substance should favour mucoid strains at the expense of non-mucoid strains by inhibiting the opsonizing effect of the precipitins and the complement dependent lysis of the bacterial cells. The work of *Doggett* & *Harrison* (1966, 1969a & b) and the results of the present study show that the non-mucoid strains predominate initially in CF pts., but as the colonization becomes chronic, mucoid strains predominate and this coincides with the development of a strong humoral immune response. These results are in accordance with the hypothesis suggested by *Hoiby* & *Axelsen* (1973) although they do not verify it.

Mrs *Anne Bathum* has skilfully performed the immunoelectrophoreses.

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PSEUDOMONAS AERUGINOSA INFECTION IN CYSTIC FIBROSIS

Distribution of B and T lymphocytes in relation to the humoral immune response

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The influence of chronic *Ps. aeruginosa* infection on the occurrence of bone marrow-derived lymphocytes (B cells) and thymus-derived lymphocytes (T cells) in peripheral blood has been studied in 2 groups of patients with cystic fibrosis. One group (9 patients) suffered from chronic *Ps. aeruginosa* infection in the respiratory tract and produced multiple *Ps. aeruginosa* precipitins which were demonstrated by means of crossed immunoelectrophoresis. The other group (9 patients) had never harboured *Ps. aeruginosa* in the respiratory tract and presented no demonstrable precipitins against this bacteria. The lymphocytes were examined for the presence of 2 surface markers for B cells receptor for C3 complement component (EAC rosette-formation) and surface immunoglobulins (immunofluorescent staining) and for the presence of 1 surface marker for T cells spontaneous binding of sheep red blood cells (E rosette-formation) The B:T cell ratio was nearly identical in the 2 groups of patients whereas the total numbers of B and T cells were significant higher in patients with chronic *Ps. aeruginosa* infection. The number of *Ps. aeruginosa* precipitins per serum was significant positively correlated to the total number of T cells, whereas the correlation with the total number of B cells was insignificant.

Specific cytoplasma membrane markers for the detection of bone marrow-derived lymphocytes (B cells) and thymus-derived lymphocytes (T cells) have been widely used to identify the cells associated with humoral immunity (B cells) and cellular immunity (T cells) in man (1 3 6, 8, 9 15–19) B cells have been identified by means of surface immunoglobulin, receptor for the C3 complement component and receptor for the Fc portion of IgG and T cells have been identified by means of the receptor which forms rosettes with sheep red blood cells and recently by a cytotoxicity test using special anti T lymphocyte sera (2, 18) Using these principles it has been shown that all or nearly all of human blood lymphocytes can be determined as either B or T cells (15 17) whereas none or few lymphocytes without such markers have been found (3 15)

Aiuti et al. (1973) have investigated the percentage distribution of B and T cells in various acute and subacute bacterial and viral infections, and they could not demonstrate any significant differences from normal values except in a few cases of mononucleosis where

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an increase in percentage of T cells was found.

However in another infectious disease, lepra lepromatosum, an abnormally high B T cell ratio has been demonstrated to be associated with depression of cellular immunity and a high level of circulating lepra antibodies (6 8)

Patients (pts.) with cystic fibrosis (CF) are frequently subject to chronic *Pseudomonas aeruginosa* (*Ps aeruginosa*) infection *in the respiratory tract* (5 12–14) Although animal experiments have indicated that *Ps. aeruginosa* contains substances which in general suppress cellular as well as humoral immunity (7) it has been shown that CF pts. produce a strong and differentiated humoral immune response against *Ps. aeruginosa* (5 12 13) However little is known about the cellular immune response or the distribution of B and T cells in these pts.

The aim of the present study was to investigate whether the ratio and number of B and T cells in CF are changed during chronic *Ps aeruginosa* infection. For this purpose 2 *groups of CF pts. have been investigated* one group with chronic *Ps aeruginosa* infection in the respiratory tract, producing many high titred *Ps. aeruginosa* precipitins (12) the other group free of *Ps aeruginosa* in the respiratory tract and without demonstrable precipitins against this bacteria. In these 2 groups of pts. the distribution of B and T cells has been investigated using 2 surface markers for B cells surface immunoglobulin (Ig) demonstrated by immunofluorescent staining (15) and receptors for the C3 complement component (EAC receptor) demonstrated by rosette formation with sheep red blood cells coated with anti-sheep red blood cell antibodies and human C3 complement (EAC rosettes) (15 19) T cells have been demonstrated by means of the surface marker (E receptor) with forms rosettes directly with sheep red blood *cells* (*E rosetttes*) (15 19)

MATERIALS AND METHODS

Patients

Eighteen CF pts. were included in the study All pts. are followed as out-pts. in the Paediatric Clinic TG Rigshospitalet, Copenhagen, the centre of CF treatment in Denmark. The diagnostic criteria have been described previously (12) The pts. have been followed, on an average, for 5 6 years (range ½ year–11 years) being examined in the clinic once a month or every other month, including bacteriological examination of the sputum or tracheal secretion as described elsewhere (14)

The criteria on which pts. were selected for the study were 1) CF pts. with chronic *Ps. aeruginosa* infection in the respiratory tract (CF + P) these pts. should have harboured *Ps. aeruginosa* in the respiratory tract for at least 1 year at the same time producing many precipitins against this organism. At the latest bacteriological investigation before this study was commenced they should have harboured solely *Ps aeruginosa* and no other bacteria in the lower respiratory tract. 2) CF pts. without *Ps. aeruginosa* in the respiratory tract (CF — P) these pts. should not have experienced infection with *Ps. aeruginosa* in the respiratory tract. They should not have had any serious problems with other bacterial species and they should not have had any bacteria in the lower respiratory tract at the latest bacteriological examination before this study was commenced.

The distribution of age and sex of the pts. are given in Table 1 and 2. At the time of this study, all the CF + P pts. presented mucoid strains of *Ps. aeruginosa* in the lower respiratory tract and no other species None of the CF — P presented *Ps. aeruginosa* in the respiratory tract, but 2 of these pts. (no. 1 & 3) presented *Staphylococcus aureus* and 1 pt. (no 5) presented *Haemophilus influenzae* in the sputum at the time of this study

Blood samples from each pt. were obtained simultaneously for the examinations of B and T cells, total counts and differential counts of peripheral white blood cells and the occurrence and numbers of *Ps. aeruginosa* precipitins. On each test-day both CF + P and CF — P were examined blindly and, as a control of the techniques, blood from the same normal control person was examined simultaneously The values obtained from this control person were always within the range of normal values given below The results of the B and T cells investigation and of the electrophoreses were obtained independently by the 2 authors without knowing the results of the other investigation. The reference values of normal control persons obtained in this laboratory using the described methods are (mean ± standard deviation) B cells (EAC) 34 per cent ± 11 B cells (Ig) 25 per cent ± 9 T cells 63 per cent ± 8

Examination for B and T cells was done according

ing to the principles of *Jondal et al.* (1972) and *Stjernswärd et al.* (1972) with some minor modifications:

Lymphocyte Separation

Ten ml of freshly drawn venous blood stabilized with heparin without preservatives (330 i.u. heparin per 10 ml blood) was diluted with 10 ml Hank's balanced salt solution (HBSS) pH 7.2, and 5 ml was layered on 3 ml of Lymphoprep solution (Nyegaard & Co. Oslo, Norway) and spun at 400 × *g* for 30 min. The cells were removed from the interface and washed 3 times in HBSS half of the cells were adjusted to 4 × 10^6 cells/ml in HBSS for the rosette-techniques, the other half being used undiluted for the immunofluorescence technique.

Labelling of Ig-bearing Lymphocytes (B cells(Ig))

0.2 ml of the isolated lymphocytes (1.5–3 × 10^6 cells) were incubated with one drop of a polyvalent rabbit anti-human immunoglobulin serum conjugated with fluorescein isothiocyanate (FITC) (Dakopatts, Copenhagen, Denmark) at 4° C for 30 min and then washed 3 times in HBSS. The cells were resuspended in HBSS and one drop of the suspension was mounted on a glass slide and covered with a cover slip.

The preparations were immediately read in a Leitz Ortholux fluorescence microscope equipped for incident light illumination using an HBO 200 mercury lamp, a heat absorption filter, a 4 mm BG 38 glass filter, a 1 mm BG 12 glass filter, a yellow glass filter (λ>480 nm), a 490 nm broad band interference filter and a TK495 dicroic mirror for excitation light selection, and a 1.5 mm 530 nm glass filter as barrier filter. The microscope had phase contrast condenser and objectives for simultaneous phase contrast microscopy in visible light. The results were read at 1000 × magnification, and 100 cells with the morphological appearance of typical small lymphocytes as judged by phase contrast microscopy were counted. Only cells showing 2 or more definite spots on the surface were counted as positive.

Sheep Red Blood Cells (SRBC)

SRBC were stored at 4° C in Alsever's solution 1:1 (v/v) and used within 8 days after bleeding.

EAC-binding Lymphocytes (B cells(EAC))

Each test-day SRBC were washed 3 times in HBSS and a 10 per cent (v/v) solution was made in HBSS. Five ml of this solution was mixed with 5 ml amboceptor (rabbit anti-SRBC, Wellcome Reagents Ltd., Beckenham, England) diluted 1:2000 (v/v), the mixture was incubated at 37° C for 30 min. The cells (EA cells) were washed 3 times in HBSS and resuspended in HBSS to a volume of 5 ml. Five ml of normal serum as source of complement (fresh serum stored at −80° C) diluted 1:20 (v/v) with HBSS was added and the suspension was incubated at 37° C for 30 min after which the suspension invariably showed some degree of haemolysis as an indication of complement fixation. The cells were finally washed 3 times and adjusted to a 1 per cent solution with HBSS. 0.25 ml of the prepared sheep cells (EAC cells) was then mixed with 0.25 ml of the (10^6) lymphocytes in a plastic tube and spun at 200 × *g* for 3 min and left at room temperature for 30 min. The cells were then resuspended by vigorous mixing (to brake E rosettes) and 1 drop of the cell suspension was mounted on a glass slide and covered by a cover slip. Two hundred lymphocytes were counted using phase contrast microscopy and 400 × magnification, and all lymphocytes binding 3 or more SRBC were counted as rosettes (EAC rosettes).

E-binding Lymphocytes (T cells)

On each test-day just before use, SRBC were washed 3 times in HBSS and adjusted to a 1 per cent (v/v) suspension in HBSS. 0.25 ml of this solution was mixed with 0.25 ml of the (10^6) lymphocytes in a plastic tube and incubated at 37° C for 15 min. The mixed cell suspension was spun at 200 × *g* for 5 min and then incubated at 4° C overnight. The top layer of the pellet was very gently resuspended by shaking, and 1 drop of the cell suspension was mounted on a glass slide, and covered by a coverslip. Two hundred lymphocytes were counted using phase contrast and 400 × magnification, and all lymphocytes binding 3 or more SRBC were counted as rosettes (E rosettes).

Precipitins against Ps. aeruginosa

Sera from the pts. were investigated by means of crossed immunoelectrophoresis for the occurrence and number of *Ps. aeruginosa* precipitins by means of a polyvalent standard antigen (St Ag) as described previously (12) with the following modifications: the crossed immunoelectrophoreses were carried out in microtechnique (on 5 × 5 cm glass plates) using agarose gel (Indubiose A 37, Batch F F 4354, L'Industrie Biologique Française S.A.) in barbital buffer pH 8.6, ionic strength 0.02 in the gel and wicks and 0.05 in the buffer vessels. 2 µl St-Ag was run against pts.' sera in the gel (15 µl pts.'s serum per cm²).

Statistical Methods

The Mann-Whitney test and Spearman's correlation coefficient R (4).

TABLE 1 *Surface Markers on Lymphocytes from Cystic Fibrosis Patients with* Pseudomonas aeruginosa *Infection*

CF + P Sex	Age (years)	Number of *Ps aeruginosa* precipitins	White blood cells per µl	Lymphocytes per µl	Lymphocytes with EAC-receptor % (= B cells (EAC))	Lymphocytes with membrane immunoglobulin % (= B cells (Ig))	Lymphoc… with E-ro… % (= T c…
1 M	7	28	9000	3600	34 %	19 %	60 %
2 M	8	22	7700	3000	46 %	40 %	63 %
3 M	9	49	11300	5420	40 %	28 %	60 %
4 M	10	20	14700	5000	47 %	33 %	47 %
5 F	11	18	8000	3600	46 %	22 %	62 %
6 F	12	21	7400	3850	30 %	10 %	56 %
7 M	13	51	14300	4720	36 %	28 %	72 %
8 M	13	40	9300	4280	39 %	25 %	72 %
9 M	14	13	10500	2000	39 %	16 %	63 %
Mean	10.5	29	10240	3940	42 %	25 %	62 %
Range	7–14	13–51	7400 –14700	2000 –5420	30–54 %	10–40 %	47–72 %

Percentage of blood lymphocytes with surface markers for B and T cells and the number of Pseudomonas aeruginosa precipitins in peripheral blood from 9 cystic fibrosis patients with chronic *Pseudomonas aeruginosa* infection in the respiratory tract (CF + P). Each patient is indicated by a number and the sex as well as age. The total number of white blood cells as well as of lymphocytes in peripheral blood is also given. For abbreviations used see text.

TABLE 2. *Surface Markers on Lymphocytes fr m Cystic Fibrosis Patients ot Harbouring* Pseudomonas aeruginosa

CF — P Sex	Age (years)	Number of *Ps aeruginosa* precipitins	White blood cells per µl	Lymphocytes per µl	Lymphocytes with EAC-receptor % (= B cells (EAC))	Lymphocytes with membrane immunoglobulin % (= B cells (Ig))	Lymphocyt… with E-recep… % (T cells)
1 M	7	0	11900	2620	35 %	23 %	72 %
2 M	8	0	7300	2920	44 %	13 %	53 %
3 F	9	0	4100	1720	34 %	17 %	75 %
4 M	10	0	4100	1760	32 %	10 %	67 %
5 F	10	0	7800	3350	31 %	20 %	52 %
6 M	11	0	6600	3170	56 %	27 %	71 %
7 F	12	0	4700	2490	39 %	7 %	55 %
8 M	12	0	3500	1440	40 %	45 %	72 %
9 M	14	0	5000	2650	33 %	33 %	
Mean	10	0	6110	2460	38 %	22 %	63 %
Range	7–14	0	3500 –11900	1440 –3350	31–56 %	7–45 %	52–75 %

Percentage of blood lymphocytes with surface markers for B and T cells and the number of *Pseudomonas aeruginosa* precipitins in peripheral blood from 9 cystic fibrosis patients who had never harboured *Pseudomonas aeruginosa* in the respiratory tract (CF — P). Each patient is indicated by a number and the sex as well as age. The total number of white blood cells as well as lymphocytes in the peripheral blood is also given. For abbreviations used see text.

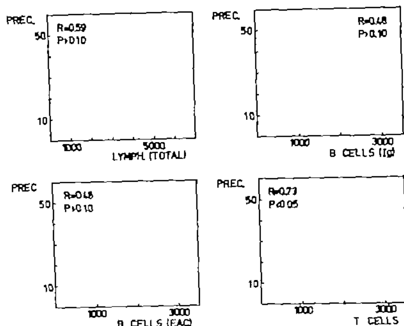


Fig. 1. Relationship between the number of *Pseudomonas aeruginosa* precipitins (PREC.) and 1) the total number of lymphocytes per µl blood (LYMPH. (TOTAL)), 2) the number of lymphocytes with membrane-bound immunoglobulin per µl blood (B CELLS(Ig)), 3) the number of lymphocytes with EAC receptor per µl blood (B CELLS(EAC)), and 4) the number of lymphocytes with E receptor per µl blood (T CELLS) in peripheral blood from 9 patients with cystic fibrosis and chronic *Pseudomonas aeruginosa* infection in the respiratory tract. Spearman's correlation coefficient R and the corresponding p values are given in the figures.

RESULTS

The results obtained in the 2 groups of pts. included in the study are given in Tables 1 and 2. It is seen that all the CF + P pts. presented multiple precipitins against *Ps. aeruginosa* whereas none of the pts. in the CF — P group had such precipitins.

It also appears from the tables that the CF + P group presented a higher total number of white blood cells than the CF — P group ($p<0.01$). There were no significant differences between the 2 groups as regards the differential counts, but the total number of lymphocytes as well as of neutrophil granulocytes were significantly higher in the CF + P group compared to the CF — P group ($p<0.01$).

With respect to the percentage of B cells (EAC), B cells (Ig) and T cells any significant differences between the 2 groups were not found, and the ratio B cells (EAC) : T cells as well as the ratio B cells (Ig) : T cells were similar in the 2 groups of pts.

According to these results, the total number of B cells (EAC) was higher in the CF + P group (mean 1640, range 780–2350 per µl) than in the CF — P group (mean 950, range 560–1770 per µl) ($p<0.02$) and similar results were found with respect to B cells (Ig) (CF + P mean 1000, range 320–1750 per µl, CF — P mean 520, range 170–890 per µl, $p<0.05$) as well as T cells (CF + P mean 2430, range 1300–3400 per µl, CF — P mean 1580, range 790–2210 per µl, $p<0.01$).

As *Ps. aeruginosa* probably is the predominating immunizing bacteria in CF + P and each precipitin specificity against the antigens of St-Ag possibly represents one separate clone of antibody producing cells, it might be interesting to see whether any correlations existed between the number of precipitins and the different subpopulations of lympho-

cytes. In Fig 1 the number of *Ps aeruginosa* precipitins per serum are correlated to the total number of lymphocytes, the number of B cells (EAC) the number of B cells (Ig) and the number of T cells. It appears from the figure that the number of T cells is significantly positively correlated to the number of precipitins, whereas the correlations between the number of precipitins and the total number of lymphocytes as well as the total number of B cells (EAC as well as Ig) were not significant. There was no significant correlation between the number of precipitins and the total number of neutrophil granulocytes.

DISCUSSION

Results reported in the literature (3 15 17) as well as those obtained in this laboratory (unpublished results) using double-labelling technique (fluorescence labelling and simultaneous rosette-formation) show that E rosettes are reliable markers for T cells, none or very few E rosette-forming cells showing markers for B cells. B cells with EAC receptors and B cells with surface immunoglobulins are, according to results reported in the literature and those obtained in this laboratory (unpublished results) mainly overlapping, most B cells having both markers (17)

It appears from Tables 1 and 2 that the percentage of T cells + the percentage of B cells (EAC) on an average is little above 100 per cent, whereas the percentage of T cells + the percentage of B cells (Ig) on an average is less than 100 per cent. The results obtained in this study therefore seems to indicate that all, or nearly all of the lymphocytes from the CF pts. could be determined as either B or T cells, a higher number of lymphocytes showing detectable EAC receptors than detectable surface immunoglobulins. Lymphocytes without the examined surface markers seems to be rare or absent.

Contamination of the lymphocytes with small monocytes covered with cytophilic antibodies which cannot be distinguished morphologically from lymphocytes (9 15 16) possibly accounted for a small percentage of the B cells. Such monocytes might bind anti-human immunoglobulin, EAC cells and EA cells (18) (the presence of which cannot be excluded, as purified IgM amboceptor has not been used in this study) the result being a slightly over-estimation of the percentage of B cells (EAC as well as Ig)

The results show that *Ps. aeruginosa* infection in CF pts. does not change the ratio B cells (EAC) T cells or the ratio B cells (Ig) T cells significantly and thus, in this respect *Ps aeruginosa* infection in CF is different from lepra lepromatosum (6, 8) and our results seems similar to those obtained by *Arati et al.* (1973) concerning other infectious diseases. During chronic *Ps. aeruginosa* infection the B cells as well as the T cells are increased in number Simultaneously the pts. produce many and high-titred (12) precipitins against *Ps. aeruginosa*. The infection with *Ps aeruginosa* and the products and constituents of this bacteria (7) have therefore no significant influence on the B T cell ratio in CF pts. as measured by the methods used in this study

The humoral immunity against *Ps. aeruginosa* seems not to be significantly correlated to the number of circulating B cells (EAC) or B cells (Ig) On the other hand, it was a surprice to find a significant correlation between the number of circulating T cells and the humoral immunity expressed by the number of *Ps aeruginosa* precipitins. This correlation is interesting and 2 possible interpretations might be offered. First, the correlation between the T cells and the number of precipitins might simply reflect a non-specific response to the infection, pts. with a higher number of precipitins presenting more severe infections than pts. with few precipitins. If this is true, it is perhaps surprising that solely T cells and neither B cells nor neutrophil granulocytes were significantly correlated to the number of precipitins. Secondly the correlation might suggest that T cells are also involved in the specific immune response against *Ps. aeruginosa* in CF pts. as helper cells for humoral immune response

and/or as mediators of delayed type hypersensitivity. Whether one of the 2 interpretations offered is true cannot be determined on the basis of the results obtained in the present study or those reported in the literature (5, 10), but must await further investigations on the cellular immunity in CF pts., including in vitro stimulation of lymphocytes with phytohaemagglutinin and with *Ps. aeruginosa* antigens, as well as migration inhibition tests.

A recent report on chronic *Ps. aeruginosa* infection in the respiratory tract of a non-CF pt. demonstrated high levels of circulating antibodies against *Ps. aeruginosa* and simultaneously diminished cutaneous delayed type hypersensitivity against *Ps. aeruginosa* and other antigens (11). It should be very interesting to study whether similar conditions are present in CF + P in spite of high levels of circulating T cells, as our results solely concerns the quantity of T cells and not the quality or function of the cells.

Mrs. *Anne Bethsen* has skilfully performed the immunoelectrophoreses.

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TABLE 2. *The Affinity of Antibodies against NIP cap in NIP HSA tolerant Mice*

Challenged with			
$NIP_{27}BSA$		$NIP_{4-1}OA$	
tolerants	controls	tolerants	controls
3 64§	2.76	3.65	10.07
5.36	2.72	10 73	25 11
2.74	4 76	4 83	18.6

§ × 10 M^{-1}
The figures are K_o values of the pooled secondary response sera to NIP-cap determined by the ammonium sulphate precipitation method. Each line represents one determination series.

observed between the two antigens (Table 3)

As individual mouse serum samples were not large enough for affinity and specificity studies an experiment was performed with rats. They were given 760 rad irradiation and subsequent multiple injections of NNP HSA. 20 days after stopping the tolerance treatment the rats were bled and challenged with alum-precipitated NNP HSA. Before the challenge the tolerant group had a higher HSA binding capacity than the control group ($p < 001$) although the antibody levels were too low for accurate determinations. No anti-NNP antibodies were found (Fig. 1) After challenge the anti-NNP antibody titre rose in the controls to ten times higher levels than in the control group. Anti-HSA antibodies remained at a low level in the tolerant group ($p < 001$) (Fig. 2) After second challenge with the same antigen the difference in anti-hapten antibodies between the tolerant and control groups was near hundred fold. A third challenge did not increase the difference but gave slightly higher antibody titres both in the tolerant and control groups. A fourth challenge did not change the titres appreciably During a subsequent 23 weeks rest period the antigen binding capacity dropped to a tenth. The tolerant animals had still low antibody levels ($p < 001$) Anti-NNP antibodies did not rise to control levels in a further challenge. 15 weeks later the rats were challenged with $NNP_{11}CG$ to see, if there still would be diminished responsiveness in the tolerant group A significant difference could be observed ($p < .001$) One year had then elapsed since stopping the tolerance inducing treatment. Anti-NNP antibodies were measured also using ^{125}I labelled NNP HOP lysine with essentially identical results.

Antibody affinities were measured by the ammonium sulphate precipitation method using NNP HOP lysine coupled with ^{125}I as the radioactive hapten. To confirm that the antibodies bind the NNP part of the hapten 9 sera were tested for inhibition of NNP HOP lysine-^{125}I binding by DIP-cap (3,5-diiodo-4-hydroxyphenylcaproic acid) as DIP is the residue that is likely to be formed, when the HOP residue is iodinated. According to unpublished experiments of O Mäkelä substoichiometric iodination of HOP results in a mixture of DIP and HOP and very little

TABLE 3 *Class Distribution of Anti-NIP Antibodies in Tolerant and Control Mouse Sera*

	Challenged with			
	$NIP_{27}BSA$		$NIP_{4-1}OA$	
	tolerant	control	tolerant	control
I challenge				
IgM	4 72 ± 17	5 13 ± 10	5 63 ± 12	5 79 ± .09
IgG	4 62 ± .28	4 98 ± 13	6.06 ± 16	6.30 ± 11
II challenge				
IgM	5 33 ± 15	6.42 ± .27**	6.66 ± .07	7 70 ± 11***
IgG	5.61 ± .20	6.98 ± .25***	7 72 ± 13	8.18 ± .08*

Log_{10} titres of anti-NIP antibodies ± standard error determined by the NIP T bacteriophage inactivation method. The significance of the difference between tolerants and controls is indicated as in Table 1

*p

with NNP-cap. Similarly there was antibody against NNP if NNP-cap inhibited NNP HOP-lysine binding more than NIP-cap. If the distinction could not be made the specificity was considered to be equal. By this technique the presence of NIP-specific and NNP-specific antibodies simultaneously in a serum could be revealed.

The ammonium sulphate precipitation method described by *Stupp et al.* (33) was used for determination of antibody affinity to NIP and to NNP The technique was modified by *measuring* the radioactivity from the precipitate rather than from the supernatant. The formula 1/b = 1/K(Ab) c + 1/(Ab) was used for plotting the results (30) *Selected rat sera were studied also by equilibrium* dialysis. For the dialyses globulin fractions were prepared from the sera by adding an equal amount of −15 C cold saturated ammonium sulphate to *the serum, mixing and centrifuging rapidly The* supernatant was discarded and the initial volume restored with distilled water The fraction was diluted judging from the measurement of antibody *quantity by the Farr assay 0 1 ml of the diluted* fraction was inserted to a small chamber with a dialysis membrane and dialyzed twice against 0.2 molar phosphate buffer in the chamber The equilibration *was carried out against 1 to 50 × 10^{-8}* molar concentrations of NNP-cap mixed with NNP HOP-lysine in a constant proportion. After overnight equilibration at room temperature (21–23 C) *the radioactivity was counted from both* fluid compartments. Similar globulin fractions were made of normal rabbit serum to obtain a correction coefficient for unspecific hapten binding The binding *by normal globulin was measured 5 times at* the standard dilutions used and it was subtracted from the binding obtained with immune sera. Then the results were plotted as before.

*IgM/IgG class distribution of anti-NIP anti*bodies was studied by NIP-conjugated bacteriophage inactivation as has been described earlier (23) In the method 2-mercaptoethanol and free *NIP-cap were used for inactivation of IgM and* IgG antibodies respectively

RESULTS

A group of mice rendered tolerant to NIP$_{14}$MSA were bled 20 days after stopping the tolerance inducing treatment. Only insignificant hapten binding was observed (Table 1) The mice were then challenged *with NIP$_{27}$BSA or NIP$_{14}$OA or NNP BSA* or BSA. If challenged with NIP$_{27}$BSA, no difference in antibody quantity could be observed between tolerant and control mice. When stimulated with NIP$_{4}$ OA there was a clearly diminished anti-NIP response in the tolerant group (p < 005) After secondary challenge with the same antigens the difference between the NIP tolerant and control groups was greater than after the first challenge. A marked tolerance effect could then be seen also in the group challenged with NIP$_{27}$BSA (p < 01) Thus a clear tolerance could be *induced* with a hapten-homologous albumin conjugate.

Anti-NNP antibodies were measured by binding of radioactive NNP BSA. Despite the 4.5 × 10 fold excess of unlabelled BSA in the test the sera from mice immunized with BSA showed an increased binding of NNP BSA over prechallenge levels (Table 1) This means that the groups having high anti-BSA titre have erroneously high anti-NNP values. Despite this bias the results *show that the* secondary challenge with NNP BSA gave low anti-NNP titres in the tolerant group *(p < 005) Nonspecific* immune depression was excluded by equal anti-BSA titres in the tolerant and control groups. This suggests that the NIP$_{14}$MSA treatment had made the mice partially tolerant to NNP also. The affinity of anti-NNP antibody was low in the NIP tolerant group, as two independent measurements of K_o values *for NNP* gave the results 8.8 and 9 0 × 10^8 1/M for pooled tolerant sera and 23 and 37 × 10^8 1/M for the controls by the *ammonium* sulphate precipitation method. This suggests that the paralysing regime with NIP$_{14}$MSA caused tolerance of the highest affinity anti-NNP antibody producing cells. Thus a proportion of the high-affinity antibodies have weak distinguishing capacity for related haptens.

The average intrinsic *association* constant K_o for NIP-cap binding was determined three times from pooled tolerant and control group sera using *the* ammonium sulphate precipitation method. The tolerant mice had slightly lower affinity antibodies than controls when challenged with NIP$_{14}$OA but not when challenged with NIP$_{27}$BSA *(Table 2)* There were no significant differences in the IgM/IgG ratio between the control and tolerant groups, although a *difference* could be

TABLE 6. *Specificity of Anti-NNP Antibodies as Determined by Simultaneous Binding of NIP-cap and NNP-HOP-lysine Iodinated with 125I in the Presence of NIP-cap or NNP-cap*

sera	N125IP-cap binding inhibited by			NNP HOP lysine-125I binding inhibited by			N125IP-cap/NNP HOP-lysine-125I binding ratio ($\bar{x}\pm$SE)
	NIP-cap	equally	NNP-cap	NIP-cap	equally	NNP-cap	
controls							
I challenge	11	0	0	1	2	8	0.62 ± .04
II challenge	2	9	0	0	0	11	0.39 ± .05
III challenge	0	7	4	0	0	11	0.39 ± .05
IV challenge	0	5	6	0	0	11	0.39 ± .05
V challenge	0	9	0	0	0	9	0.48 ± .07
after rest period	1	8	0	0	0	9	0.40 ± .08
last challenge	0	9	0	0	0	9	0.41 ± .05
tolerants							
III challenge	0	17	0	1	16	0	–
V challenge	5	9	0	0	7	7	0.69 ± .09
after rest period	0	9	1	0	7	3	0.63 ± .07
last challenge	3	7	0	0	3	7	0.61 ± .06

The figures refer to the number of sera classified in each group. The binding ratio has been calculated from the bindings obtained, when both haptens are added to the tubes in a 1 × 10⁻⁸ molar concentration.

(Table 6). In the secondary response this heteroclitic component mainly disappeared and only a normal crossreaction was observed. *The ratio of NIP binding capacity/* NNP-binding capacity in the presence of both haptens was *significantly greater in the* primary response sera than in the later sera ($p < .01$). In the tolerant group the specificity of the antibodies was heterogeneous throughout the study. Many sera showed NIP-specific components and some sera had a higher binding capacity for NIP than for NNP.

DISCUSSION

The results show that *hapten* specific tolerance can be obtained by injecting into animals great amounts of hapten coupled to homologous or to heterologous albumins. The tolerance obtained in mice with NIP coupled mouse serum albumin was remarkable in that a primary challenge with an immunogenic conjugate resulted in nearly normal antibody levels. Still the secondary challenge with the immunogen showed, that the animals were unable to mount a normal secondary response. This was not due to inefficient T cell participation, as NIP on ovalbumin carrier also showed the diminished anti-hapten antibody response. The results suggest, that the B cells in the NIP MSA tolerant mice were able to respond to stimulation by antibody production but were unable to proliferate to a memory cell population.

The primary anti-BSA response was significantly higher in the tolerant than in the control groups. The same phenomenon was observed also when the mice were challenged primarily with NNP-BSA (Table 1). This might be due to increased likelihood for the antigen to meet BSA-specific cells in the absence of hapten specific cells. Another explanation could be that the animals were primed to new antigenic determinants shared by NIP MSA and NIP BSA. This resembled the situation observed earlier in NIP BSA induced tolerance, where the mice were primed to BSA while tolerant to NIP (31).

The rats paralyzed with *NNP HSA* did not mount a primary response when challenged with the same conjugate. The anti carrier antibody response was weak suggesting that the T cell helping effect was missing.

Again the secondary response was absent. The rats got thereafter multiple challenges with the antigen. The tolerant group always had smaller antibody titres than the controls. The last challenge with NNP on a heterologous carrier showed again a low anti NNP titre. This suggests that the B cell tolerance was partly irreversible in contrast to the findings of *Chiller et al* (8). Also *Howard* (17) has shown, that B cell tolerance may be partly irreversible. *Davie et al* (9) have shown that two months after DNP D-GL induced tolerance the highest affinity antibody secreting cells were still missing. Thus it seems likely that part of the B cell tolerance may be long lasting. *Fidler & Golub* (11) have shown, that the tolerogen given in an immunogenic form and dose during the vanishing period of tolerance is capable of prolonging the unresponsive state. It is possible that the multiple challenges have influenced the duration of the tolerance in this work. This is, however probably not responsible for the prolonged tolerance, because the rest periods in the immunization were so long as to cause a total disappearance of the tolerance if the timetable published by *Chiller et al.* (8) should be universal.

The remaining antibody production in B cell tolerance has been found to be of low affinity (34 36 37). In this study the tolerant mice challenged with NIP$_{4.4}$OA produced low affinity antibodies. The tolerant mice challenged with NIP$_{27}$BSA, however did not show diminished antibody affinity when compared to the controls. The affinity was lower than that obtained by NIP$_{4}$ OA challenge. This could be explained by the fact that highly coupled hapten protein conjugates, like the NIP$_{27}$BSA used in this study have been found to be partially thymus-independent antigens (1). On the other hand it has been shown that T cell participation is important for high-affinity antibody production (13 19). Thus the lack of T cell effect is the probable reason for the weak tolerance expression in the groups stimulated with NIP$_{27}$BSA.

There are conflicting reports showing that the antibody specificity either decreases (14, 25) or increases (6 35) with increasing affinity. The work done on this point by *Gershon & Kondo* (14) may be criticized. They used the crossreaction of sheep and horse red blood cells as indicator of antibody specificity. Continuing immunization with sheep red blood cells resulted in antibodies crossreacting with horse red blood cells indicating diminishing specificity. *McBride & Schurman* (27) have shown that antibody to one determinant on chicken red blood cells is able to exaggerate the antibody response to another determinant, that is normally not immunogenic. A related phenomenon might be due to the occurrence of crossreacting antibody in *Gershon & Kondo's* experiment. This study anyhow showed elimination of the heteroclitic antibody population during maturation of the immune response (Table 6). The affinity changes towards different haptens, that happen with change in structure of the antigen binding site, may proceed differently. With haptens binding to the antibody in the ionized stage such as NIP (20) the location of the ionic charge in the protein may be important. If the charge moves from a suboptimal position to optimal position, both the specificity and the affinity will increase simultaneously. The nitrophenols used by *Little et al* (25) are relatively hydrophobic. Increasing hydrophobicity of the binding site might then result to increased affinity and increased binding also of other hydrophobic haptens as was observed. The results available on the relationships of affinity and specificity might fit to the view that the specificity change in maturation is due to chance but affinity increase important. That is: In a maturation process the affinity towards the haptenic determinant is by definition critical to the selection of the antibody producing cell clones. In a normal immunization with haptens there are probably seldom question of crossreactions with proteins of the organism's own. Thus antibodies with specificity crossreacting with another hapten are neither favored nor of disadvantage. Specificity changes are therefore limited only by

the processes eliminating autoimmune cell clones. The divergent results of studies concerned in antibody specificity in the process of immune maturation are thus explainable in a simple way

The tolerance treatment did not change the distribution of anti-hapten antibodies into IgM and IgG classes. This may be used as an argument suggesting, that there are no fundamental differences in the arrangement of IgM and IgG receptor antibodies of B cells. *Hraba et al.* (18) have noticed a diminished IgG plaque forming cell response in tolerant animals, but the result may have been due to isolated T cell tolerance as well. *Fidler & Golub* (11) have shown that trinitrobenzene sulfonic acid causes tolerance of mainly indirect plaque forming cells. *Katsura* (22) has studied the tolerance to BSA. With low antigen doses both IgM and IgG antibodies were suppressed. Large antigen doses gave a small IgG response, which was unexplained. Thus there are few studies available where the effect of T cell tolerance is strictly excluded when B cell tolerance class has been studied.

The results obtained in this work seem to indicate, that an anamnestic secondary type response can not be obtained in B cell tolerance because the proliferation of specific B cells is inhibited. The tolerance involves not only IgG receptor antibody carrying cells but also IgM receptor carrying cells.

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INDIRECT IMMUNOFLUORESCENCE STUDIES OF SMOOTH MUSCLE ANTIBODIES

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The reactions of IgG smooth muscle antibodies were studied in an indirect immunofluorescent (IIF) staining system. In chessboard titrations it was found that a conjugate with molar F P ratio of 3.6 gav a plateau titre which was the same as, or higher than that of a conjugate with a molar F P ratio of 2.2. The plateau endpoint concentration of antibody protein in the conjugates fell within the range from 6.3 to 12.5 mcg/ml. The antibodies were species- and organ-non-specific, and they could be absorbed with a crude actomyosin extract prepared from human uterus or human kidney Differences in antigenic strength of different tissues occurred Rat stomach smooth muscle and rat vessel walls yielded higher plateau titres than rat renal glomeruli. Fixation of the tissue sections gav lower titres than unfixed sections. The antibodies were unable to fix complement in a complement IF system.

Antibodies reactive with smooth muscle components have been demonstrated by the immunofluorescent method in sera from patients with lupoid hepatitis (9) acute infective hepatitis (6) infectious mononucleosis (7) and in certain malignant diseases (16)

In the present study reactions of sera which gave a staining pattern typical of smooth muscle antibodies were studied by the immunofluorescent method. In order to evaluate a standardized immunofluorescent system, chessboard titrations were performed with different conjugates and different tissues. By means of a complement immunofluorescence technique, the capacity of antibodies to fix complement was also studied. In addition, absorption experiments were performed in order to study some of the characteristics of the antigen reacting with these antibodies.

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MATERIALS AND METHODS

Sera

Six human sera with smooth muscle antibodies (SMA) were studied four were from patients with clinical and laboratory signs of liver disease one was from a patient with suspected infectious mononucleosis, and one was from patient in whom the diagnosis was unknown. The last-mentioned serum contained SMA in low titre as well as antinuclear antibodies. Liver biopsy had been performed in two of the patients with liver disease. Histological examination revealed posthepatitic cirrhosis in one case and macronodular cirrhosis in the other Furthermore, two sera without SMA were included in the study one contained gastric parietal cell antibodies and the other mitochondrial antibodies the latter was from a patient with primary biliary cirrhosis.

Antigens

Tissues from the stomach and kidney of rats and guinea pigs and from the human uterus were used as antigens. Human tissue was obtained from specimens removed at operation, and animal tissue was removed immediately after the animals had been killed by ether anaesthesia. Tissue blocks were frozen in liquid nitrogen and stored at −70 C until use. For immunofluorescence, tissue sections, 4 μ in thickness were cut on a cryostat, air-dried and used immediately or stored in a freezer for up to one week. The tissue sections were used without fixation or after treatment for 5 min with one of the following solutions 4 per cent neutral formalin, 35 per cent ethanol, or acetone. After fixation the sections were washed in phosphate-buffered saline (PBS) for 10 min.

Indirect Immunofluorescent Staining (IIF)

Sera and conjugates used for the IIF were diluted in PBS with 2 per cent bovine serum albumin (BSA) For the washings, the buffer was used without BSA. Normal human serum (NHS) and PBS/2 per cent BSA were included in each titration as negative controls; the positive sera were used in doubling dilutions, starting with 1 10 The titrations were carried out as described earlier (1)

In the chessboard titrations, each serum dilution row was treated with serial dilutions of the conjugate The same serum dilution rows were used for titrations with different conjugates in order to avoid differences in titres due to variation in the serum dilutions.

Complement Immunofluorescent Staining (CIF)

Sera, conjugates and complement used for CIF were diluted in a barbital buffer at pH 7.3 (11) Normal human serum was used as complement source. Fresh normal human blood without anticoagulants was collected, allowed to clot and centrifuged. Serum aliquots of 2 ml were prepared and stored in a freezer (−70 C) The CIF was carried out as described earlier (1)

In order to establish the necessary amount of complement for CIF chessboard titrations were performed with the normal human serum which was used as complement source, and a complement-fixing mitochondrial antibody-containing serum. Both sera were used in two-fold dilutions The antihuman C4 conjugate was used diluted 1:32. The "plateau endpoint of complement" corresponded to a dilution of 1 16 in one experiment and to a dilution of 1 8 in a second experiment. For the CIF titrations, the normal human serum used as complement source was employed at a dilution of 1:4

Conjugates

The fluorescein-labelled conjugates are kindly supplied by Professor *E. H Beutner* Department of Microbiology State University of New York at Buffalo, USA. The characteristics of the conjugates are listed in Table 1 The protein content of the conjugates was determined by the biuret method, and the fluorescein concentration by the method of *McKinney et al.* (12) The antihuman IgG conjugates contained antibodies to light as well as heavy chains. They did not react with IgM and IgA in gel precipitation. The antibody contents of the conjugates were determined by a reverse immunodiffusion method (2) For the CIF an antihuman C4 conjugate was used. This conjugate had an antibody content of 16 units per ml (3). The antigen for this unitage determination was undiluted normal serum.

Microscopy

The slides were examined with a binocular fluorescence microscope (Zeiss) with a HBO 200 mercury vapour lamp and a dark-field condenser. The exciter filter was an interference filter (400–505 nm) adjusted for fluorescein isothiocyanate and the barrier filter (Zeiss 50) was matched to the interference filter (13)

Absorption Experiments

Tissue homogenates from human uterus and kidney were used for the absorption experiments.

TABLE 1 *Characteristics of Four Goat Antihuman Conjugates*

Characteristic	Conjugate No			
	419	309	310	375
Specificity	Anti IgG	Anti IgG	Anti IgG	Anti C4
Molar F P ratio	3.6	2.2	4.2	2.1
Antibody protein Concentration (mg/ml)	4.1	1.8	3.4	

* The antibody content of the antihuman C4 conjugate was 16 units per ml.

The human uteri were surgical specimens obtained from fertile women on whom hysterectomy had been performed because of abnormal uterine bleeding. The kidneys were obtained from autopsies and were macroscopically normal. The tissue was homogenized and extracted with a histidine buffer with 1mM ATP (N) (14). The resulting tissue extract was centrifuged at 5000 rev/min for 5 min to remove ATP-insensitive material and then lyophilized. The dried powder was used for absorption of sera. In other experiments, the tissue was extracted with histidine buffer without ATP or with PBS. These extracts were lyophilized and the resulting powder used for absorption. The absorption was carried out as follows. Sera were diluted 1 10 with PBS/2 per cent BSA and the tissue powder added. The mixtures were kept at room temperature for 1 hour under continuous shaking and then left 4 C overnight. The next day the mixtures were centrifuged (2500 rev/min, 10 min) and the supernatant was examined for antibodies by IIF

RESULTS

The antibodies studied exhibited an immunofluorescent staining pattern which corresponded to the distribution of smooth muscle in tissue. They reacted with components of human myometrium as well as with stomach and kidney tissue from the rat and guinea pig. The antibodies are referred to as smooth muscle antibodies (SMA) In rat stomach (RS) they reacted with the outer muscular layer the muscularis mucosae and strands of tissue between the gastric glands. Staining of the vessel walls (RV) was observed both in stomach and kidney In kidney sections staining of components of the renal glomeruli (RG) probably the capillary walls (8) was also observed. Figs. 1 and 2 show positive reactions with rat stomach and rat kidney

Fixation Experiments

SMA titres obtained with fixed rat tissue sections were lower than those obtained with unfixed tissue sections. Similar results were found when human myometrium was used as antigen For this reason, tissue sections without fixation were employed for further experiments. The results are shown in Table 2.



Fig 1 Staining of muscularis mucosa and smooth muscle strands between glands in rat stomach section.



Fig 2 Staining of vessel walls and renal glomerulus in rat kidney section.

TABLE 2. *Effect of Fixation of Rat Stomach and Kidney Sections Used for Demonstration of SMA*

Antigen	Fixation: None			Neutral formalin 4 %			Ethanol 35 %			Acetone		
	RS	RG	RV	RS	RG	RV	RS	RG	RV	RS	RG	RV
X-44	40	20	40	<10	<10	<10	<10	<10	20	10	<10	[illegible]
X 126	160	80	320	40	<10	40	80	40	160	80	40	80

RS, rat stomach smooth muscle RG rat renal glomeruli RV vessel walls in rat kidney

TABLE 3. *Plateau Titres and Plateau Endpoints of the Conjugate in Chessboard Titrations with Three Smooth Muscle Antisera on Different Tissues*

Serum		Conjugate 419 Tissue			Conjugate 309 Tissue			Conjugate 310 Tissue		
		RS	RG	RV	RS	RG	RV	RS	RG	RV
6177	PT	40	10–20	40	40	20–40	20–40	40	20	40
	PEP	12.5	12.5	12.5	6.3	6.3	6.3	12.5	6.3	12.5
X 15	PT	80	40–80	80	80	40–80	80	N.D	N.D.	N.D
	PEP	12.5	12.5	12.5	6.3	6.3	6.3	N.D	N.D.	N.D
X-44	PT	160	40	80	80	20–40	80	N.D	N.D.	N.D
	PEP	12.5	12.5	6.3	12.5	6.3	12.5	N.D	N.D.	N.D

RS rat stomach smooth muscle RG rat renal glomeruli RV vessel walls in rat kidney PT plateau titre PEP plateau endpoint N.D., not done.

Chessboard Titrations

The results of six chessboard titrations performed with serum X-44 two different conjugates, and three different antigens are shown in Fig 3 It is seen that the titres were constant over a range of conjugate dilutions, i.e. they were plateau titres. At lower concentrations of conjugate the titres fell abruptly or stepwise. The highest conjugate dilution which still gives the plateau titre, is called the plateau endpoint (3)

Using smooth muscles from rat stomach (RS) as antigen, it is seen from Fig 3 that serum X-44 yielded a plateau titre of 160 with a conjugate with a molar F P ratio of 3 6, and a plateau titre of 80 with a conjugate with a molar F P ratio of 2.2. Using rat glomeruli (RG) as antigen, the plateau titres with the two conjugates were 40 and 20–40 respectively A plateau titre of 80 was obtained with both conjugates when vessel walls of rat kidney (RV) were used as antigen. These differences in plateau titres could not be attributed to variation in serum or conjugate dilutions as the same dilutions were used in all six experiments.

Table 3 shows the plateau titres and the plateau endpoint of conjugates found in chessboard titrations with three human sera and three conjugates on different antigens. It is seen that the plateau endpoints were 12.5 mcg per ml in 12 of the 21 chessboard titrations in the remaining nine cases the plateau endpoints were 6.3 mcg per ml.

It is also seen from Table 3 that stomach smooth muscle (RS) and vessel walls (RV) yielded approximately the same titres, while renal glomeruli (RG) gave lower titres than the two former antigens. This cannot be explained by differences in serum dilutions or differences in F P ratios of the conjugates as this phenomenon occurred in experiments

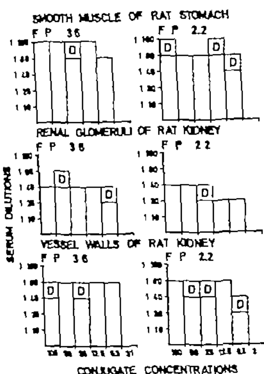


Fig. 3 Chessboard titration with SMA-containing serum, two antihuman IgG conjugates with different F P ratios and three different antigens. Conjugate concentrations in mcg per ml. D = doubtful.

in which the same serum dilution rows and the same conjugate had been used.

Complement Fixation

The ability of smooth muscle antibodies to fix complement was examined by CIF. Mitochondrial antibodies are known to fix complement (1 15). Therefore, a mitochondrial antibody-containing serum was included in each titration as a positive control. The mitochondrial antibody titre obtained in CIF and IIF were identical (320) and these titres were comparable as the molar F P ratio of the antihuman C4 conjugate and the antihuman IgG conjugate were approximately the same, 2.1 and 2.2, respectively. The IIF titres of the six SMA-positive sera were 320, 80, 80, 80, 40 and 10. The reactions obtained in CIF were either negative or doubtfully positive in a serum dilution of 1 10. Thus, SMA were not found to fix complement by CIF.

Absorption Experiments

After absorption with an ATP-sensitive material from human uterus referred to as crude actomyosin the SMA titres decreased.

TABLE 4 *Antibody Titres Before and After Absorption with Crude Actomyosin*

Serum	Antibody tested	Antigen	Unabsorbed	Absorbed with crude actomyosin: 50 mg per 0.1 ml serum	100 mg per 0.1 ml serum	150 mg per 0.1 ml serum
X-44	SMA	Stomach smooth muscle	80	40	<10	<10
6177	SMA	Stomach smooth muscle	40	10	<10	<10
X-61	SMA	Stomach smooth muscle	10	N.D	N.D	<10
X-61	ANA	Gastric parietal cells	320	N.D	N.D	160
X 72	PA	Gastric parietal cells	320	N.D.	N.D	320

SMA, smooth muscle antibody ANA, antinuclear antibody PA, parietal cell antibody N.D not done.

TABLE 5 *SMA Titres Before and After Absorption with Tissue Extracts*

Serum	Antigen	Unabsorbed	Absorbed with	
			human kidney	human uterus
X-44	RS	80	<10	<10
	RG	40	<10	<10
	RV	80	<10	<10
X-61	RS	10	N.D	<10
	RG	<10	N.D	<10
	RV	10	N.D	<10

RS rat stomach smooth muscle RG rat renal glomeruli RV vessel walls in rat kidney; N D not done.

The results of the absorptions are shown in Table 4 It is seen that relatively large amounts of antigen (i.e. 1–1.5 g/ml serum) were necessary to *abolish the staining completely In* order to examine whether this absorption effect could be due to non-immunological binding of immunoglobulins, absorption experiments were performed with a serum which contained parietal cell antibodies (PA) and a serum which contained antinuclear antibodies (ANA) and SMA. The absorption did not alter the PA titre of serum X 72. The ANA titre of serum X-61 fell from 320 to 160, but this difference cannot be considered significant.

Table 5 shows the results of absorption of an SMA positive serum either with crude *actomyosin from human uterus or a* lyophilized kidney homogenate. The kidney homogenate was extracted with histidine buffer with ATP Both tissue extracts were able to absorb the antibodies which reacted with the three antigens used for IIF

Absorption with tissue homogenates extracted from human uterus either with histidine buffer without ATP or PBS caused a decrease in SMA titre. However it was not possible to achieve complete absorption of SMA when these homogenates were employed in concentrations of 1–2 g per ml of serum, i.e. the concentrations of crude actomyosin which could absorb the SMA completely Thus, the amount of antigen in these extracts was lower than that in extracts prepared with histidine buffer with ATP

DISCUSSION

The IgG smooth muscle antibodies (SMA) *studied in the present work* were organ- and species-non specific. *Johnson et al.* (10) found sera which gave smooth muscle staining but no glomerular staining They suggested tissue-specific antibodies might occur In this work, the antibody titres obtained with renal glomeruli as antigen were lower than those obtained with the other antigens. Thus, sera which yielded low titres on stomach smooth muscle might appear negative when tested on renal glomeruli.

The antibodies could be absorbed completely with crude actomyosin from human myometrium and with a similar material from human kidney After absorption with either of these preparations the staining of rat stomach as well as of rat kidney was prevented. This could indicate that the antibodies studied react with a common antigen present in stomach smooth muscle, in vessel walls as well as in renal glomeruli. However absorption experiments with purified antigen preparations of rat stomach smooth muscle and rat renal glomeruli should be done in order to clarify this further

Relatively high concentrations of crude actomyosin (i e. 1–1.5 g per ml of serum) were required for the absorption of antibody The tissue preparation probably contains large amounts of substances other than antigen, or the antigenic sites may have been altered by the preparation procedure e g lyophiliz-

zation. Tissue homogenates prepared from human myometrium with buffers without ATP yielded a material the absorption effectiveness of which was much lower that that of the ATP-sensitive material. This might explain the findings by *Johnson et al.* (10) who failed to extract antigen with saline solution.

Pretreatment of tissue with certain fixatives reduced the titres. This is in accordance with the findings by other authors (10 17) and could be explained by dissolution or destruction of the antigen by the fixatives.

The plateau titres were dependent on the antigen used, and also on the F.P ratio of the conjugate. Thus, a conjugate with a high F P ratio gave a plateau titre which was the same as, or higher than that of a conjugate with a low F P ratio. Conjugates with very high F P ratios were not employed in this study because they give excessive non-specific staining of tissue and plateau titres which are difficult to define (1 3)

Regardless of the tissue used as antigen, the plateau endpoints of the conjugate were found to be within the range from 6.3 to 12.5 mcg per ml. Thus, an antibody concentration of 25 mcg per ml always gave the plateau titre without yielding any non-specific staining with the conjugates used. The plateau endpoints of the conjugates fell in the same range of values obtained in chessboard titrations with other antibodies, i.e. antinuclear antibodies, basement membrane antibodies, mitochondrial antibodies, and treponemal antibodies (1 3 4 5)

The use of unfixed tissue sections and conjugates with a molar F P ratio of about 4 seems to be suitable for the demonstration of SMA. The antibody concentration should be about 25 mcg per ml. The same conditions have been found to be optimal for the demonstration of antinuclear antibodies (3) bullous pemphigoid antibodies (18) and mitochondrial antibodies (1)

The IgG antibodies to smooth muscle were not found to fix complement by the CIF method, although this method was found suitable for the demonstration of complement fixing mitochondrial antibodies (1) The reason for this is not clear and this problem merits further investigation.

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INTERACTION BETWEEN C1q C1r and C1s FROM HUMAN SERUM

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The presence of Ca^{++} changed the electrophoretic mobility of C1s. Incubation of purified preparations of C1r and C1 in the presence of Ca^{++} resulted in the formation of a C1r–C1s complex, which by crossed immunoelectrophoresis was demonstrated in the β-region. In mixtures of purified C1q, C1 and C1 containing Ca^{++} a complex consisting of C1q, s was obtained, which crossed immunoelectrophoresis demonstrated in the γ-region. No complex formation was found between C1q and C1s in the absence of C1r

C1 in normal human serum is a 19S molecule consisting of 3 distinct proteins C1q C1r and C1s linked together by calcium ions (*Lepow et al.* 1963) When EDTA is added the molecule disintegrates into its three components, which can be separated by chromatographic fractionation (*Naff et al.* 1964 *Laurell & Siboo* 1966)

In the present paper crossed immunoelectrophoresis was used to study the complex formation between purified preparations of C1q, C1r and C1s under various experimental conditions.

MATERIAL AND METHODS

C1q was purified according to *Yonemasu & Stroud* (1971) The purified protein precipitated soluble antigen-antibody complexes (*Agnello et al.* 1969) and agglutinated latex particles coated with human IgG (*Ewald & Schubert* 1966)

C1 was purified according to the procedure of *Lepow et al* (1963) and dialysed against triethanol buffered saline (TBS) pH 7 4 The preparation formed hemolytically acti. C1 when added to purified preparations of C1q and C1s (*Lepow et al.* 1963) On incubation with a non-acti. C1s preparation C1s formed, as described by *Naff & Ratnoff* (1968) The preparation did not hydrolyse N-acetyl-l-tyrosine ethyl-ester and did not precipitate with specific anti-C1s or anti-C1q in gel precipitation tests.

C1s was purified according to *Haines & Lepow* (1964) and dialysed against TBS. The product obtained had esterase activity as shown by hydrolysis of N-acetyl-l-tyrosine-ethyl-ester (*Levy & Lepow* 1959 *Laurell et al.* 1965) The C1s activity was determined according to *Levy & Lepow* (1959) Non-activated C1s was prepared from EDTA euglobulin in the presence of Liquoid® (10 µg/ml) during the purification procedure. Liquoid inhibits the activation of C1s by C1 (*Naff & Ratnoff* 1968)

Antisera to C1q and C1 respectively were obtained on immunization of rabbits with purified preparations and absorption to apparently monospecificity in conventional immunoassays.

Crossed immuno-electrophoresis was performed as described by *Laurell* (1965) The primary electrophoretic separation as well as the electrophoretic running into the antibody containing gel was performed using either barbital buffer 0.075 M pH 8.6 containing Calcium 2×10^{-3} M or EDTA 2×10^{-3} M, or phosphate buffer pH 6 µ 0.1 containing EDTA 2×10^{-3} as described for each experiment.

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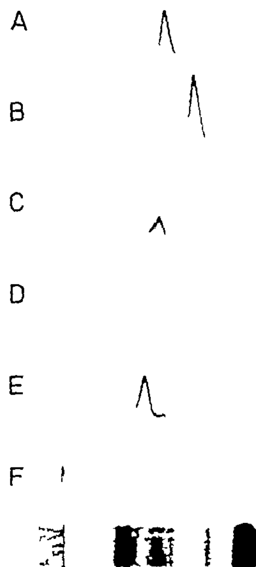


Fig 1 Crossed immunoelectrophoresis of the C1 subcomponents. The analyses were done with anti C1s in the gel in the second electrophoretic step.

A. Purified C1s. Both electrophoretic steps were run with Ca^{++} barbital buffer

B. Purified C1s. Both electrophoretic steps were run with EDTA barbital buffer

C. Mixture of C1s (final concentration 75 U/ml) and the C1r preparation in a final dilution of 1/3. Both electrophoretic steps were run with Ca^{++} barbital buffer

D. Mixture of C1s (final concentration 75 U/ml) and the C1r preparation in a final dilution of 2/3. Both electrophoretic steps were run with Ca^{++} barbital buffer

E. The same mixture as in Fig. 1 D. The separating electrophoresis was run with Ca^{++} barbital buffer and the second electrophoretic step with EDTA barbital buffer

F. Mixtures of C1s (final concentration 75 U/ml), the C1r preparation in a final dilution of 2/3 and C1q. Separating electrophoresis was run with Ca^{++} barbital buffer and the second electrophoresis with EDTA barbital buffer

RESULTS

Interaction of C1s with Calcium Ions

The appearance of purified C1s on crossed immunoelectrophoresis after electrophoretic separation in the presence of Ca^{++} and EDTA, respectively, is given in Fig 1 A and 1 B.

When the separating electrophoretic step was performed with Ca^{++} barbital buffer C1s was situated in the α region more cathodically than when EDTA-barbital buffer was used for the separating step.

Interaction between C1s and C1r

Crossed immunoelectrophoresis against anti C1s was performed with a mixture in Ca^{++} barbital buffer of C1s (final concentration 75 U/ml) and the C1r preparation in a final dilution of 1/3. With Ca^{++} present during both the electrophoretic steps two peaks appeared, one in the α region in the same place as C1s when run alone in the presence of calcium, the other in the β region (Fig. 1C). The β peak was not distinct but stained within the contour indicating a complex, probably between C1s and C1r. When a mixture containing C1r in a final dilution of 2/3 was used only the β peak appeared, indicating that all C1s was now complexed with C1r (Fig. 1 D).

Under the same experimental conditions as described in the legends to Fig. 1 D but with EDTA present during the second electrophoretic step the β peak was distinct and increased in height indicating a dissociation of C1s from the C1r–C1s complex when Ca^{++} was removed (Fig. 1 E).

No peak was visible in the β region when

the separating electrophoresis was done with EDTA barbital buffer

Interaction between C1q C1r and C1s

When crossed immunoelectrophoresis against anti C1q was performed on purified C1q with phosphate buffer pH 6, containing EDTA, C1q formed a precipitate in the slow γ region. When the Ca^{++} barbital buffer was used for the separating electrophoresis and EDTA phosphate buffer for the second electrophoretic step the precipitate appeared as a very narrow peak corresponding to the site of the application slit. No precipitate appeared when the barbital buffer was used in the second step

Mixtures of constant amounts of C1q and of C1s with various dilutions of C1r were incubated at 37 C for 30 minutes in the presence of Ca^{++} Crossed immunoelectrophoresis using anti C1s in the gel was run on the mixtures with Ca^{++} barbital buffer during the separating electrophoresis and EDTA barbital buffer during the second step With the mixture containing C1r in a final dilution of 2/3 three peaks appeared (Fig 1 F)

One was located at the application site and migrated forwards to the positive pool, and represented C1s dissociating from the C1q, C1r C1s complex. One peak appeared in the β region, as shown for the C1r-C1s complex. The third peak appeared in the α_2 region at the normal site of C1s, when run alone. The precipitate in the β region representing C1r-C1s complex did not appear with a mixture containing the C1r in a final dilution of 1/3 indicating that all the completed C1r-C1s was included in the C1q, r s complex. With anti C1q in the gel and EDTA phosphate buffer in the second step one precipitation peak appeared at the application site and migrated towards the cathode. This means that the material present at the application site contained C1q as well as C1s.

Interaction between C1q and C1s

C1q and C1s were incubated in the presence of Ca^{++} and crossed immunoelectrophoresis was performed against anti C1s with Ca^{++} barbital buffer during the separating electrophoresis and with EDTA barbital buffer during the second step. No C1s peak was found at the application site and there was no change from the normal position of C1s in the α region and the height of the C1s peak was normal. This finding indicated that C1q did not complex with C1s in the absence of C1r

DISCUSSION

The results showed that C1s migrated slower in the presence than in the absence of Ca^{++} and thereby indicated a change in the charge of C1s on binding of calcium ions. Other components of complement behave in the same way in the presence of Ca^{++} C3 migrates as a slow β globulin when run in Ca^{++} containing buffer but with roughly the same electrophoretic mobility as transferrin when Ca^{++} is omitted (*Laurell et al.* 1956) Using ultracentrifugation analysis *Valet & Cooper* (1973) recently found a dimer formation of purified C1s in the presence of calcium ions.

The double peaked precipitate with C1s antigenic determinants, appearing when a mixture of C1s and C1r was analysed with Ca^{++} present during the electrophoresis indicated that the β precipitate represented a complex between C1s and C1r The filled area within the contour of the β peak lends further support to the assumption that this precipitate is produced by a complex (*Ganrot* 1972) When calcium was omitted during the separating electrophoresis no such complex formed. We have not succeeded in raising an antiserum to C1r of sufficient titre to definitely prove this assumption. However our findings are in line with those briefly reported by *Valet & Cooper* (1973) who used ultracentrifugal analysis and demonstrated complex formation between C1r and C1s.

On agarose electrophoresis at pH 8.6 C1q did not enter the gel whether calcium ions were present or not, but it did appear in the

slow γ region when the electrophoresis was performed with EDTA phosphate buffer at pH 6 This agrees with earlier observations on the electrophoretic mobility of C1q (*Muller Eberhard* 1968 *Yonemasu* & *Stroud* 1971) The complex between C1q C1r and C1s was also found at the application site when the electrophoresis was run with the Ca^{++} barbital buffer When appropriate amounts of the subcomponents C1q C1r and C1s were mixed, C1s appeared as three molecular species as a molecule in the α_2 region probably as a dimer (Fig 1 A) as a complex with C1r in the β region, and in a complex consisting of C1q C1r and C1s at the application site.

The finding of C1q as well as of C1s at the application site, on analysis of the mixture of C1q C1r and C1s in the presence of Ca^{++} and of C1s situated in the α_2 region when C1r was not included in the mixture supported the view that C1q and C1s do not complex in the absence of C1r No evidence of complex formation between C1q and C1r was produced by ultracentrifugal analysis by *Valet* & *Cooper* (1973)

The interaction between the C1 subcomponents has been thoroughly studied. Efforts were made to elucidate the mechanism of the activation of the C1s subcomponent to an esterase on binding of C1 to an immune complex (*Loos et al.* 1972) In a brief report *Sakai* & *Stroud* (1973) confirmed the finding of an activation of C1s by C1r by *Naff* & *Ratnoff* (1968) *Valet* & *Cooper* (1973) isolated C1r from serum in a proenzyme form. On trypsin treatment the purified proenzyme was activated and then capable of activating C1s. The purification method used by us gave C1r in the activated state.

Further studies on the interaction between the subcomponent of C1 and on the conditions during which C1r and C1s are activated may help to widen the knowledge of diseases in man. Preliminary data obtained in our laboratory on plasma and sera from patients with chronic urticaria show that in some of these patients circulating complexes of C1r and C1s occurred, often in association with an imbalance between the concentrations of C1q and C1s (*Laurell et al. to be published*)

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BRIEF REPORT

PURIFICATION BY IONIC PRECIPITATION OF THE FIRST COMPONENT OF HUMAN COMPLEMENT AND COMPLEMENT ESTERASE

G von Zeipel, Hanna-Stina Hanson and L. V von Stedingk

The first component of complement (CI) was purified from euglobulin by precipitating at a pH of 7.5 and an ionic strength of 0 115 μ for two cycles at +4 C followed by one cycle at +22 C. From euglobulin prepared by acid precipitation, CI was purified more than 8 times with a recovery of 60–70 per cent. From euglobulin precipitated at a neutral pH, CI of a similar degree of purity was obtained with a recovery of 70–80 per cent. After EDTA treatment and low ionic precipitation (0 0? μ) such preparations of CI yielded fractions containing CI esterase purified about 20-fold with respect to the esterase activity of euglobulin.

Materials and Methods

Serum. Fresh sera from blood donors were either processed directly or after some weeks of storage at −75 C.

Euglobulin. For acid precipitation of euglobulin (acid EU) according to *Lepow et al.* (1963) 200 ml of serum was diluted 1 8 under magnetic stirring with cold 0.02 ionic strength acetate buffer of pH 5.5 containing 0 15 mM $CaCl_2$. The mixture was left at 0 C for 18 hours until centrifuged at 15000 *g* for 30 min. The precipitate was dissolved in 30 ml of 0.005 M phosphate buffer pH 7.5 containing 0.3 M NaCl and 0 15 mM $CaCl_2$. The solution was centrifuged at 15000 *g* for 30 min The supernatant was treated as described under Results.

For neutral precipitation of euglobulin (neutral EU) according to *Tamura & Nelson* (1968) and *Loos et al.* (1970) 200 ml of serum adjusted to pH 7.5 was diluted 1 5 with cold 0 005 M phosphate buffer containing 0 15 mM $CaCl_2$. The mixture was stirred at 0 C for 30 min. until centrifuged at 15000 *g* for 30 min. The precipitate was dissolved in 15 ml of 0.005 M phosphate buffer pH 7.5 containing 0.3 M NaCl and 0.15 mM $CaCl_2$. The solution was centrifuged at 15000 *g* for 30 min. In some experiments the supernatant at this stage was further treated as described under Results. In other experiments the supernatant was reprecipitated at 0 C by dilution 1 30 with 0 005 M phosphate buffer pH 7.5 containing 0 15 mM $CaCl_2$. The resultant precipitate, twice washed with the same buffer and dissolved and centrifuged as above was further treated as described under Results.

Complement esterase (CIs) was quantitated by measuring the hydrolysis of N-acetyl-L-tyrosine-ethyl-ester (ATEe) by pH stat titration at 37° C (see *von Zeipel* 1972) The reaction volume of 5 ml contained the specimen to be tested and final concentrations of 0 0? M ester 0.005 M phosphate buffer pH 7.5 and 0.2 M NaCl. One unit of esterase is that amount which liberates 0 5 micromoles of H in 15 min.

Clq, IgG and IgM were assessed on EDTA-treated samples by single radial immunodiffusion (*Mancini et al.* 1965) in plates containing 1 per cent agarose 10 mM EDTA, 0 05 M Tris-glycine buffer pH 8 and 0 15 M NaCl. Antisera from Behringwerke Germany were mostly used at 1 per cent concentration. IgG and IgM were also determined in LC-Partigen plates from this company

Protein measurements were made with the Folin phenol reagent (*Lowry et al.* 1951) Protein in fractions containing EDTA, which is known to interact seriously with this reagent (J 1972) was measured after precipitation by trichloroacetic acid (*Chase & Williams* 1968)

All buffer solutions contained 0.01 per cent sodium azide as preservative

Results

The following procedures have so far been applied to eight different preparations of euglobulin, with highly reproducible results.

Step 1 Cold 0.005 M phosphate buffer pH 7.5 with 0.15 mM $CaCl_2$ was added dropwise to an

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contain any detectable amounts of C1q, IgG and IgM. The content of C1r could unfortunately not be tested. The sediments containing most of C1q at a 6- and 12 fold purification were further subjected to a gradient centrifugation in sucrose (10 to 40 per cent) at 0.5 M NaCl (*Sledge & Bing* 1973). The gradient centrifugation resulted in an additional twofold purification of C1q, with removal of IgM among other proteins. The preparation of C1q, however, still contained small amounts of IgG.

Although the same schedule was successfully applied to the purification of C1, C1s and C1q present in neutral and acid EU, these starting materials showed some dissimilarities. In neutral EU C1 was present as activated complex (C1). On the contrary, C1 in acid EU had to be activated at 37° C for 15 min (*Lepow et al.* 1958). However, such an activation of C1 in acid EU took place without temperature-treatment when the ionic strength in Step 1 was lowered from 0.315 μ to 0.115 μ. In Step 1, the recovery of C1 from acid EU was usually 10 to 15 per cent lower than that from neutral EU. This could, however, be compensated for (not shown in the table) by collecting a second precipitate formed during storage of the centrifuged supernatant of this step for an additional 24 hours at 4° C. From the amount of C1 present in the Step 1 material of acid or neutral EU, generally 90 to 95 per cent, was recovered in Step 2. Between Step 2 and Step 3, an additional 5 to 10 per cent of C1 was usually lost. A recovery of more than 100 per cent between Steps 1 and 2 was for unknown reasons obtained in the experiment on neutral EU recorded in the table.

Comments

In the present investigation, C1 was purified from human euglobulin by precipitation at the highest possible ionic strength which still gave a good recovery. An ionic strength of 0.115 μ was found to be optimal in this respect, i.e. a salt concentration about threefold higher than that used to precipitate euglobulin (and C1) from human serum (*Lepow et al.* 1963, *Tamura & Nelson* 1968). The procedures enabled a rapid treatment of large quantities of material to give preparations of C1 of moderate purity. Highly purified C1s was easily obtained from these.

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order to obtain cells containing sufficient amounts of hyaluronidase the bacteria were harvested about 30 min after the exhaustion of glucose.

All centrifugations were performed at 20,000 × *g* for 10 min at 4 °C, unless otherwise stated.

Measurement of cell disintegration. The optical density of cell suspensions in buffer was determined by measuring the absorbance of suitably diluted samples in a Zeiss PMQ II spectrophotometer at 550 nm. Determination of optical density was made prior to and after the disintegration procedure and the estimation of cell disintegration was expressed in ΔO.D. i.e. the difference between these determinations. Determinations were made in duplicates. Immediately prior to the determinations of optical density the cell suspension was homogenized by six strokes in a tissue grinder (A. H. Thomas, Philadelphia, U.S.A.) The relation between optical density and dry weight of these cells has been investigated in a previous study (4) It was found that the relationship

$$\frac{A_{550}}{\text{mg dry weight per ml}} \quad \text{was } 3.2$$

Assay of hyaluronidase activity Hyaluronidase activity was determined as previously described (4)

Assay of aminopeptidase activity The determination of aminopeptidase activity was made by a modification of the method of *Goldberg & Rutenburg* (3) as described by *Linder et al.* (5)

Materials Hyaluronic acid grade III-S was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Reagents for the determination of aminopeptidase activity were purchased from Sigma. Chloramphenicol was obtained from Park Davies.

Standard method for disintegration by freeze-pressing The so-called X-press (Biotec) described by *Edebo* (2) was used for disintegration. The cells were washed once in 10–20 ml cold (4–7 °C) 0.01 M phosphate buffer (pH 6.8) and after centrifugation resuspended in 20 ml 0.05 M phosphate buffer (pH 6.8) supplemented with chloramphenicol (CAP) 100 μg/ml. The cell density (A_{550}) was usually between 12 and 36, corresponding to 4 and 11 mg dry weight of cells per ml. After homogenization and determination of optical density the suspension was transferred to an X-press (X 25 maximum capacity 25 ml) that had been kept at -30 °C for at least 10 hours. After six hours at -30 °C the cells were pressed through the orifice of the X-press once. The cell material was then immediately removed from the press and thawed at 7 °C. The suspension was homogenized and measured for optical density and then centrifuged. The enzymatic activities of the supernatant were determined within 20 hours after the cells had been transferred to the X press.

Standard method for disintegration by autolysis. The cells were washed and then resuspended in 0.05 M phosphate buffer supplemented with CAP as described above. The cell density of the suspension was usually approximately 4 mg/ml (dry weight) Homogenization and determination of optical density of the suspension were performed as described. The cell suspension was then incubated at 37 or 7 °C for 20 hours. At the end of the incubation period, the suspension was homogenized, its optical density measured, and then centrifuged. Enzyme activities of the supernatant were determined immediately

Calculations The specific enzyme activity was calculated by the formula

$$C = \frac{E}{\Delta O.D.}$$

where *C* is the specific enzyme activity *E* is enzyme activity per ml of supernatant and ΔO.D. is the difference between the optical density of the cell suspension before and after the disintegration either by freeze-pressing or by autolysis.

RESULTS

Release of hyaluronidase and aminopeptidase by autolysis at 37° C and at 7° C and by freeze pressing In order to investigate the effect of time and temperature on the specific enzyme activities during autolysis and to compare the specific yields of the enzymes released by autolysis and by freeze-pressing the following experiments were performed. Samples of cells harvested at the same time were disintegrated by autolysis and by freeze-pressing The cell suspensions in buffer had an initial optical density of A_{550} = 12. Autolysis was carried out at 7 °C and 37 °C. The autolytic procedure was in accordance with the standard method except that the incubation time varied.

Autolysis, expressed as ΔO.D. is plotted against time in Fig 1 The rate of autolysis increased with the temperature. The decrease in optical density during autolysis carried out for 24 hours was about 45 per cent at 37° C and about 19 per cent at 7 °C. Freeze-pressing reduced the optical density by about 87 per cent.

The specific hyaluronidase and aminopeptidase activities in extracts prepared by autolysis carried out for various lengths of time are plotted against time in Fig 2. The specific enzyme activities were not significantly affected by the incubation temperature It is

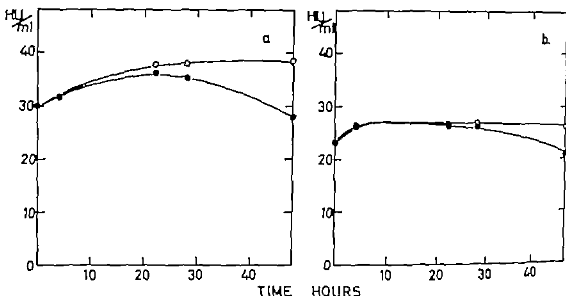


Fig 3 a and b Effect of time and temperature on hyaluronidase activity of extracts prepared by freeze-pressing The cell material was passed once (X-press 1) (Fig 3 a) or three times (X-press 3) (*Fig. 3 b*) through the orifice of the press. The mean hyaluronidase activity *of supernatants of* extracts centrifuged at 1 000 × *g* and 5,000 × *g* and 20,000 × *g* is plotted against time. The incubation temperatures were 7 C and 37 C. Hyaluronidase activity at 7 C ○——○ and 37 C ●——●

trifugations performed for 10 min at 1,000 × *g*, 5,000 × *g* and 20 000 × *g* The X press is so constructed that the frozen cell material can be passed back and forth through the orifice of the press in order to increase the disintegration of cells. The effect of repeated pressings on the disintegration of cells and on the activity and stability of the enzymes was also investigated in these experiments. The *cell material was divided into two equal* parts one part was passed through the orifice *of the press once* (X *press 1 extract*) and the other part was passed three times (X press 3 extract) The suspensions had an A_{500} = 56 corresponding to about 11 mg dry weight of cells per ml. In order to keep pH close to 6 and to give enough material for the experiment, the supernatants were diluted 10-fold with the same buffer immediately after thawing

The results obtained are shown in Figs. 3 a *and* b *and 4 a and b. No significant differ ences in* hyaluronidase activity of superna tants obtained by centrifugation at different forces could be observed. The experimental data presented in Fig 3 a and b are mean values *of the activity present in* 1,000 × *g*, 5 000 × *g* and 20,000 × *g* supernatants.

The crushing of cells by one passage through the press decreased the optical density of the suspension by about 80 per cent. Additional passages could not significantly increase the disintegration efficiency under these conditions. As can be seen by compar ing the zero time hyaluronidase *activity of Fig 3 a with that of 3 b and the zero time* aminopeptidase activities of Fig. 4 a with *those of 4 b, the additional two passages of the frozen* cell material decreased the enzyme activities of the extracts. The loss in hyaluronidase activity was about 23 per cent and the mean loss in aminopeptidase activity was about 35 per cent.

Aminopeptidase activity of the superna tants increased when the *isolation of the* enzyme was performed *at lower centrifugal forces. It may thus be concluded that amino-peptidase* activity was in part associated with particulate matter sedimentable by centrifugation at 5 000 × *g* and 20 000 × *g for* 10 min.

Both hyaluronidase and aminopeptidase

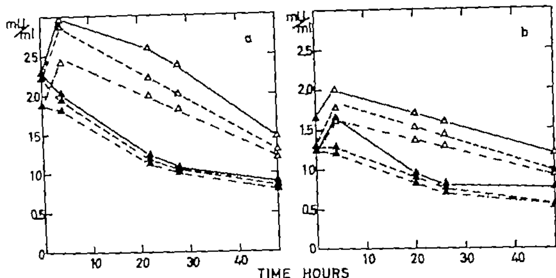


Fig 4 a and b Effect of time and temperature on aminopeptidase activity of extracts prepared by freeze-pressing. The cell material was passed once (X-press 1) (Fig 4 a) or three times (X-press 3) (Fig. 4 b) through the orifice of the press. The aminopeptidase activity of supernatants obtained by centrifugation at 1,000 × g, 5,000 × g and 20,000 × g is plotted against incubation time. The incubation temperatures were 7 C and 37 C. Aminopeptidase activity at 7 C △——△ and at 37 C ▲——▲ Supernatants obtained after centrifugation at 1,000 × g —— 5,000 × g – – – – and 20,000 × g – – –

TABLE I. *Specific Enzyme Activities in Cell-free Extracts after Autolysis in Phosphate Buffers of Various Molarities*

Molarity of buffer	C_H	C_A
0	1.2	0.3
0.01	7.3	0 6
0.02	7.9	0.6
0 05	8 2	0.7
0.1	6.2	0.6
0.5	5.9	0.8
1.0	5.6	1.0
2.0	3.5	0.8

The specific hyaluronidase activity (C_H) and aminopeptidase activity (C_A) were calculated by the formula $C = \frac{E}{\Delta O.D}$ where C is the specific activity E is enzyme activity per ml of extract and ΔO.D is the decrease in optical density during autolysis.

activity increased in most supernatants during the initial part of the incubation period. No decrease in hyaluronidase activity was observed for at least 48 hours at 7 C and for about 22 hours at 37° C. By comparison with hyaluronidase, aminopeptidase activity was unstable both at 7 C and at 37 C. Inactivation was most rapid at 37 C. Comparisons between X-press 1 and X press 3 supernatants showed that the rate of inactivation was more rapid in X-press 1 supernatants and that after 48 hours the differences in activity of X-press 1 and X-press 3 supernatants were small. The rate of inactivation in supernatants obtained after centrifugation at different forces was approximately equal.

Effects of salt concentration on autolysis and on the specific release of enzymes It has been shown earlier that the salt concentration affected autolysis in buffer (1 7) It has also been observed that the dissociation of proteins from cell walls and from ribosomes was influenced by the salt concentration (10 13)

The following experiments were performed to study the effect of salt concentration on autolysis and on the specific enzyme activities of cell-free extracts. Cells were incubated at 37 C in phosphate buffer of various molarities, pH 6.8, containing CAP 100 μg/ml.

Samples were taken at intervals and check

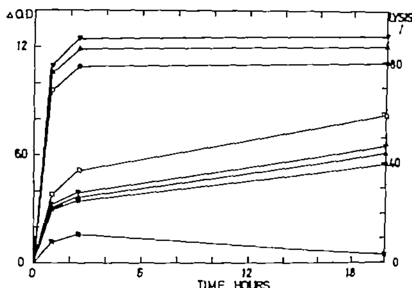


Fig 5 Effect of salt concentration on autolysis. Autolysis was carried out at 37 C in phosphate buffer pH 6.8 of various molarities. Autolysis, expressed as the decrease in optical density (ΔO.D.) and as per cent lysis is plotted against incubation time. The cell suspensions had an initial optical density of 14.0±0.1 Buffer molarity OM ■——■ 0.01 M ●——● 0.02 M ▲——▲ 0.05 M —— 0.1 M □——□, 0.5 M ○——○ 1.0 M △——△ 2.0 M ——

ed for optical density at 550 nm. After 20 hours of incubation the suspensions were homogenized and centrifuged at 20,000 × *g* for 10 min. The supernatants were diluted to a final salt concentration of about 0.01 *M* before the enzyme activities were determined. The specific enzyme activities, corrected for the dilutions, are recorded in Table 1

From Fig 5 it can be seen that the rate of autolysis was related to the salt concentration. The rate of autolysis increased with increasing salt concentration. By the following experiment it was also found that the capacity to autolyse was not significantly decreased by incubation in low molarity buffer for 20 hours. The pellets obtained by centrifugation at the end of the 20 hours incubation period were resuspended, to the same optical density as initially in 0.1 M phosphate buffer pH 6.8 containing CAP 100 μg/ml and incubated at 37 C for a further 20 hours. Cells that had previously been incubated in salt concentration less than 0.1 M continued to autolyse to a final optical density of the suspension slightly above that of the 0.1 M suspension after the first period of incubation. Cells that had previously been incubated in buffers of 0.1 *M* or higher salt concentration did not autolyse during this second period of incubation.

The experimental data presented in Table 1 show that the specific hyaluronidase activity was maximal in 0.05 M buffer extract. The specific activity then decreased with increasing salt concentration. The specific yield of aminopeptidase increased when the salt concentration was above 0.1 M and was maximal in 1.0 M buffer

Effect of pH on autolysis. Autolysis in 0.05 M phosphate buffer carried out for 1 hour at 37 C at pH-values ranging from 5.8 to 8.0 was maximal in the pH-range 7.4-7.7

The effect of CAP on the extraction of aminopeptidase The addition of CAP to the buffer solution was made in order to inhibit "de novo" synthesis of the enzymes during autolysis and during the freezing and thawing procedures of the X-press method. In a work on formation of the enzymes (6) it was shown that CAP at a concentration of 100 μg/ml completely inhibited the synthesis of hyaluronidase in media otherwise permitting hyalu-

advantageous or even necessary when more unstable enzymes are to be investigated.

The release of "soluble" aminopeptidase during autolysis was directly proportional to cell lysis within a wide range of values of ΔO.D and was independent of the incubation time and temperature. Two important criteria for an intracellular marker suggested by *Pollock* (9) seem to be fulfilled by aminopeptidase activity of this strain since aminopeptidase was found to be released proportionally to cell lysis and to be stable in cell suspensions.

The specific yield of hyaluronidase obtained by autolysis carried out for 4 hours was high compared to the yields after longer incubation time. Inactivation of the enzyme during incubation or incomplete washing of the cells prior to the suspension in phosphate buffer are explanations that both seem less probable. It seems more likely that the homogenization procedure, performed at the end of the incubation period, released hyaluronidase from cells of the initial stage of cell wall lysis. This theory was supported by the observation that, when this homogenization was omitted the specific yield of hyaluronidase was lower after 4 hours of incubation than after longer incubation time. However homogenization was necessary to obtain reproducible results.

The observation that hyaluronidase of this strain was was sensitive to freezing and thawing was in agreement with the finding of *Soder* & *Nord* (12) who reported that hyaluronidase isolated from dental plaque material was inactivated by freezing and thawing.

It was shown that the aminopeptidase activity in extracts prepared by freeze-pressing was unstable both at 37 C and at 7 C, which did not seem to occur in extracts prepared by autolysis. This observation emphasized the importance of highly standardized procedures when the results of comparative experiments are based on quantitation of aminopeptidase activity in extracts prepared by freeze-pressing. It is possible that the instability of aminopeptidase was due to the freezing and thawing procedures. As both the increase and decrease of aminopeptidase activity ran parallel in extracts obtained after centrifugation at different forces (Fig. 4) the aminopeptidase of the different fractions seemed to have the same stability characteristics. Hyaluronidase activity remaining after the freeze-pressing procedure showed little inactivation.

Lysis in buffer of whole cells and cell walls in many different organisms has been reported by a number of investigators. The buffer salt concentration affected lysis which was usually maximal in buffers of 0.01–0.05 molarity. Thus *Streptococcus faecalis* (11) *Bacillus psychrophilus* (7) *Bacillus subtilis* (14) and *Lactobacillus acidophilus* (1) showed maximal lysis in buffers of 0.01–0.05 molarity. Lysis of *Bacillus cereus* (8) was maximal in 0.05–0.1 M buffer and *Escherichia coli* (8) in 0.2–0.4 M.

Further investigations are necessary to explain the finding that the rate of autolysis increased with increasing salt concentration of the phosphate buffer. The salt concentration may have affected lysis by osmosis or by ionic strength. The ionic strength of the buffer may have influenced an autolytic enzyme system directly or affected the dissociation of autolytic enzymes from sites where they were inactive. Salt concentration affected the specific aminopeptidase activity most probably by increasing the dissociation of aminopeptidase from particulate matter at high concentrations of salt. The specific effect of CAP on the yield of aminopeptidase in 20,000 × g supernatants of extracts may be explained by increased detachment of aminopeptidase from particulate matter. To my knowledge, such an effect of CAP has not been reported earlier. It was observed that CAP also increased the yield of aminopeptidase from autolysing cells in proteose-peptone medium in a similar concentration dependent manner (6). It is evident that such an effect must be taken into account in investigations of enzyme synthesis and release when CAP is used to inhibit protein synthesis.

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ESTIMATION OF CELL LYSIS DETERMINATION OF AMINOPEPTIDASE IN EXTRACTS OF *STREPTOCOCCUS MITIS*, ATCC 903

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A colorimetric method has been tested for the determination of aminopeptidase in crude extracts of *Streptococcus mitis*. This was done in order to evaluate the possibility of using this enzyme as an intracellular marker in studies concerned with the quantitation of cell lysis. No purification of the enzyme was applied since the determination of cell lysis by an intracellular enzyme marker is dependent on the accurate and rapid measurement of the enzyme activity in supernatants from autolyzing cultures. Under the assay conditions used the enzymic reaction proceeded at constant rate, proportional to enzyme concentration over a wide range. The rate of the enzymatic reaction was maximal at 35–40° C. The optimum pH for activity was 7.2. The enzyme was stable in the pH range 5.2–9.8. The rate of the reaction was decreased at high substrate concentration.

Bacterial enzymes may be classified into extracellular intracellular and ectocellular (6) according to their site of action. The extracellular enzymes are liberated from undamaged cells and are accumulating in the extracellular medium, while the intracellular enzymes are found in cell extracts. The ectocellular enzymes are considered to be located at the cell surface, in a so-called periplasmic space between the cell wall and the cytoplasmic membrane. These enzymes may be selectively released by osmotic shock of the cells as described by *Heppel* (5) but are normally found in the cell extract. Bacterial cells are commonly prone to autolyze when exponential growth has ceased. Cell bound proteins, both truly intracellular and those located in the periplasmic space are then liberated into the extracellular growth medium. However also during exponential growth, a fraction of cells may undergo lysis which is often insufficient to significantly affect the net increase in cell turbidity Consequently cell-bound enzymes may occur in the extracellular medium both in stationary phase cultures and in cultures growing exponentially It is evident that conclusions regarding location of certain enzymes in normally dividing cells, based on their occurrence in the extracellular medium, may not be drawn without proper control of the degree of cell lysis. The quantitation of cell lysis by measurement of some intracellular product in the extracellular medium was suggested by *Pollock* (13) This author used α-glucosidase as an intracellular marker in *Bacillus subtilis*.

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Peptidases seem to have some advantages as intracellular markers in bacteriological work. 1 It is generally believed that peptidases (exopeptidases) contrary to proteases (endopeptidases) are truly intracellular enzymes in most bacteria. The intracellular character of aminopeptidase in strains of *Escherichia coli* has been established by *Neu & Heppel* (11) and in *Bacillus subtilis* by *Mimamura et al* (10) and in *Mycobacterium phlei* by *Plancot et al.* (12) 2. Peptidases seem to be widely distributed among bacterial species (17) Although most studies on bacterial peptidases have been performed on strains of *Escherichia coli* and *Bacillus* the occurrence in strains of *Neisseria* (1) *Aeromonas* (14) and *Micrococcus* (9) have been reported. In a recent study the occurrence of aminopeptidase in *Streptococcus mitis* was demonstrated (7) 3 Bacterial peptidases are believed to be constitutive. This suggestion was based on studies on peptidase in *Escherichia coli* by *Simmonds & Toye (15)* These investigators showed that bacteria growing in the absence of exogenous peptides had the same level of peptidase activity as cells growing in the presence of peptides.

The aim of the present investigation was to study the accuracy of a colorimetric method for measuring aminopeptidase activity in extracts of *Streptococcus mitis* and to evaluate optimal conditions regarding pH, temperature, substrate concentration and time for the enzymic reaction.

MATERIAL AND METHODS

Strain Streptococcus mitis ATCC 903

Cultivation technique The cells were cultured in a stirred fermentor at constant pH under anaerobic conditions. The nutrient medium was a glucose-proteose-peptone medium, previously described (7)

Preparation of cell extract The cells were harvested by centrifugation about 120 min after logarithmic growth had ceased and washed once in cold (4 7° C) 0.01 M phosphate buffer at pH 6.8 containing chloramphenicol, 100 µg/ml, to a concentration of cells corresponding to 40 mg dry weight per ml. Disintegration of the cells was performed by freeze-pressing according to *Edebo* (3) After the freezing, crushing and thawing procedures the suspension was centrifuged at 5,000 × g for 15 min at 4 C.

The supernatant fluid, stored at 4 C, was used as stock enzyme preparation. Samples were taken from this preparation and diluted in 0.05 M phosphate buffer pH 6.8, immediately prior to use.

Nearly all enzymes that hydrolyse L-leucyl-β-naphthylamide have been designated leucine aminopeptidase. *Smith & Rutenburg* (16) have suggested the name amino acid napthylamidase for human tissue enzymes that hydrolyse this substrate, whereas *Delange & Smith* (2) have suggested the name aminopeptidase for crude enzyme preparations. As crude enzyme preparations were used we will refer to it simply as an aminopeptidase activity of cell extracts of *Streptococcus mitis*.

Reagents for the determination of enzyme activity was purchased from Sigma Chem. Co St Louis, Mo., USA. Chloramphenicol was obtained from Park Davies. Other chemicals used were of reagent grade.

Enzyme Assay Procedure

The determination of enzymatic activity was made according to a method developed by *Goldberg & Rutenburg* (4) for determination of leucine aminopeptidase activity in urine and serum. Some minor modifications of the method were made, as described below 0 2 ml of 0.00685 M L-leucyl-β-naphthylamide hydrochloride solution was mixed with 1.3 ml 0.1 M phosphate buffer pH 7.2. 0.5 ml of the diluted enzyme preparation was then added. The enzyme-substrate mixture was incubated for two hours at 37 C. The enzymatic reaction was terminated by the addition of 1.0 ml of perchloric acid solution (20 per cent w/v) to the enzyme-substrate solution. 1 0 ml of this mixture was then transferred to another test tube and one tablet of sodium nitrate (2 mg) was added. After 10 min at 37 C, the excess of sodium nitrite was decomposed by addition of 1.0 ml of a 0.5 per cent (w/v) ammonium sulphamate solution. After another two min, 2.0 ml of 0 05 per cent (w/v) N-1-naphthyl-ethylenediamine dihydrochloride solution was added to yield a blue azo-dye. This reaction was developed for 30 min at 37 C. The absorbance at 578 nm was measured in a Zeiss (PMQ II) spectrophotometer after a further 60 min at room temperature. The studies of the dependence of pH, temperature, time and substrate concentration were performed as described above, with appropriate modifications, (variable pH, temperature, time and substrate concentration) All rates were corrected for spontaneous hydrolysis. All values are based on the average of at least duplicate assays.

One unit of aminopeptidase activity is defined as the amount of enzyme which liberates 1 µmole β-naphthylamine per min at 37 C when the concentration of L-leucyl-β-naphthylamide is 0.685 mM.

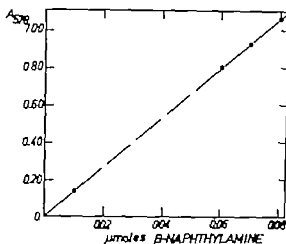


Fig 1 Calibration curve prepared by the measurement of diazotized β-naphthylamine. The absorbancy of the azo dye at 578 nm as a function of µmoles β-naphthylamine.

Preparation of Calibration Curve

Solutions of 1.0 ml consisting of 0–0.08 µmoles of β-naphthylamine in 0.1 M phosphate buffer were prepared from a standard solution. To each of these solutions, one tablet of sodium nitrite was added and after 10 min at 37 C, 1.0 ml of ammonium sulphamate and, after another two min, 2.0 ml of N-1 naphthyl-ethylenediamine dihydrochloride were added. The various mixtures were read in the Zeiss spectrophotometer at 578 nm after 60 min at room temperature. The spectrophotometrical determinations are plotted against concentration of β-naphthylamine in Fig 1

RESULTS

The aminopeptidase activity of extracts of *Streptococcus mitis* prepared as described was so high that the enzyme samples were diluted stepwise 800 times with 0.05 M phosphate buffer pH 7.2, unless otherwise stated, to permit assay

Effect of Time on the Reaction

Samples of the diluted enzyme solution were incubated with the substrate at 37 C for various periods ranging from 30 to 300 min. The enzymic reaction was terminated by the addition of perchloric acid. The whole experimental series was then assayed for β naphthylamine release. As can be seen from Fig 2, the hydrolysis of the substrate proceeded at constant rate for five hours at the concentrations of substrate and enzyme used.

Effect of pH on the Reaction and on the Stability of the Enzyme

The effect of pH on the activity of the enzyme was studied by incubating the diluted enzyme preparation with the substrate in the following 0.1 M buffers sodium acetate-acetic acid buffer Na_2HPO_4—NaH_2PO_4 buffer and glycine—NaOH buffer The effect of pH on the aminopeptidase activity in the range 3.8 to 9.8 is shown in Fig 3 The optimum pH for activity was 7.2. No enzyme activity was found at pH below 4.8 and the activity was close to zero at 9.8.

The stability of the enzyme at various pH values was investigated in the following way A stock enzyme sample was diluted 200 times with distilled water Three volumes of this preparation were mixed with one volume of a 0.1 M buffer of suitable pH. The buffers were the same as those in the experiment described above. The enzyme preparations were kept at various pH-values ranging from 3.8 to 9.8 for 30 min at 37 C. At the end of the incubation period, 8 volumes of 0.1 M phosphate buffer pH 7.2 were added to each preparation in order to permit assay of enzyme activity at pH 7.2 It can be seen from

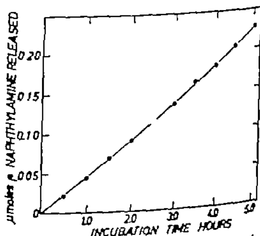


Fig. 2 Effect of time on the hydrolysis of L-leucyl-β-naphthylamide. Release of β-naphthylamine is plotted against the incubation time of the enzyme-substrate mixture at 37 C.

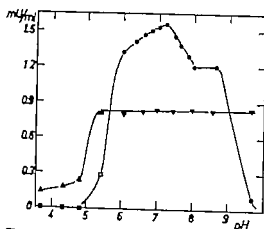


Fig 3 Effect of pH on the activity and on the stability of the enzyme. The hydrolysis was carried out at 37 C in the following 0.1 M buffers: sodium acetate-acetic acid buffer □—□, Na_2HPO_4 – NaH_2PO_4 buffer ●—● glycine–NaOH buffer ○—○.

Experiments on pH-stability were performed by incubation of the enzyme in buffers of 0.025 M final concentration at various pH-values for 30 min at 37° C. Sodium acetate—acetic acid buffer △—△ Na_2HPO_4—NaH_2PO_4 buffer ▼—▼ glycine—NaOH buffer ▽—▽ The activity of the enzyme was then determined at pH 7.2.

Fig 3 that the enzyme was not affected by pH in the pH range 5.2–9.8 under the conditions of the experiment. The experimental data presented in Fig 3 also show that the activity of the enzyme preparations stored at pH-values ranging from 5.4 to 9.8 was almost 50 per cent less than the activity of the preparation assayed at pH 7.2 without prior storage, in spite of the fact that the concentration of the enzyme preparation was the same during assay

Effect of Enzyme Concentration

The effect of enzyme concentration was studied by measuring the hydrolytic rates of various dilutions of the stock enzyme preparation. A linear relationship for enzyme samples diluted 160 320 640 1280, 2560 5120 and 10240 times was observed (Fig. 4)

Effect of Substrate Concentration on the Velocity of the Enzymatic Reactions

As the hydrolysis of L-leucyl-β-naphthylamide proceeded according to zero order

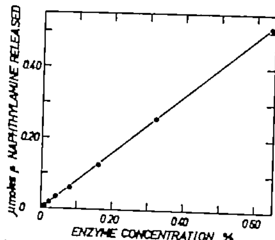


Fig 4 Effect of enzyme concentration on the rate of the reaction. The rate of hydrolysis expressed in µmoles β-naphthylamine released per 120 min is plotted against the concentration of the enzyme preparation expressed as percentage (v/v) of the stock enzyme solution.

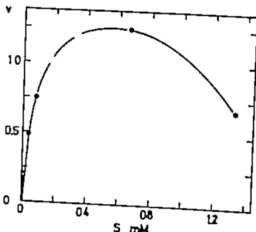


Fig 5 Effect of substrate concentration on the rate of the enzymatic reaction. The velocity of the reaction (v) expressed in terms of µmoles L-leucyl-β-naphthylamide hydrolysed per min per ml of the stock enzyme solution diluted 1000 times is plotted against the concentration of L-leucyl-β-naphthylamide.

kinetics with respect to substrate concentration for at least 300 min it may be concluded that, as regards the concentration of substrate and enzyme used, the enzyme was saturated with substrate during this time and that the reaction proceeded at maximal velocity

In order to study the effect of substrate concentration on the velocity of the enzymic

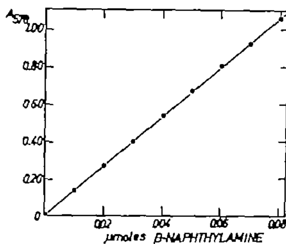


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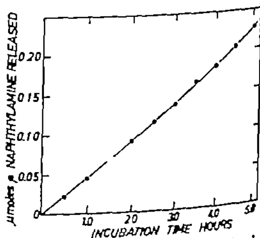


Fig 2 Effect of time on the hydrolysis of L leucyl-β-naphthylamide. Release of β-naphthylamine is plotted against the incubation time of the enzyme-substrate mixture at 37° C.

perature zone in which it has high activity (4) an analytical procedure for the measurement of the appearance of the products of the reaction.

The sensitivity of the method was very high. An aminopeptidase activity of 0.1 mU per ml of the enzyme preparation could be accurately measured. The standard method permitted assay of enzyme concentrations which catalysed the release of at least 0.500 μmoles β naphthylamine corresponding to the transformation of about 36 per cent of the substrate. The enzymatic hydrolysis proceeded at constant rate for at least 300 min, indicating the stability of the enzyme in the reaction mixture at 37 C.

Many previous reports on bacterial aminopeptidases have shown that metal ions such as Co^{++} Mg^{++} Mn^{++} and Ca^{++} activate these enzymes (5 10 12). It is possible therefore that the decrease in activity observed at high substrate concentration was due to lack of such cofactor. However studies of enzyme properties such as activation and inhibition by metal ions should not be performed on crude cell extracts. In a study now in progress it was found that partially purified *Streptococcus mutis* aminopeptidase was activated by cobalt ions.

The results obtained indicate that the method for measuring aminopeptidase activity in crude extracts of *Streptococcus mutis* is well suited for use in studies concerned with the quantitation of cell lysis.

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bound and released fractions of the enzymes at different phases of the growth cycle.

Since the results indicated a cell-bound location of hyaluronidase which was in contrast to the findings obtained by other investigators, experiments were also carried out to investigate a possible surface location permitting the enzyme in intact bacteria to act on extracellular substrates.

MATERIALS AND METHODS

Culture conditions. The cultivation of the cells was performed in a stirred culture vessel (FG 500, Biotec, Stockholm, Sweden) at 37 C under anaerobic conditions at controlled pH (6.5) The cultivation technique and the nutrient medium, called PP1 have been described in a previous article (4)

Samples were withdrawn at different growth phases and centrifuged. All centrifugations were performed at 20,000 × *g* for 10 min at 4 C.

Assay for hyaluronidase activity Hyaluronidase activity was determined as previously described (4) and expressed in Hultin units (H.U.)

Assay for amino peptidase activity Aminopeptidase activity was determined by the method described by *Linder et al.* (5) and expressed in units (U)

Determination of cell-bound activities. The sedimented cells were washed once in cold (4 7 C) 0.01 M phosphate buffer pH 6.6. Disintegration of the cells was then performed by controlled autolysis in phosphate buffer supplemented with chloramphenicol (CAP) as previously described (3) Since CAP specifically increased the aminopeptidase activity autolysis was also carried out in phosphate buffer without added CAP

Calculations. The enzymic content of the cells as calculated by the formula

$$C = \frac{E}{\Delta O.D}$$

here C is the specific enzyme activity E is enzyme activity per ml of the supernatant of the cell extract and ΔO.D is the decrease in optical density during the autolytic procedure.

Total cell-bound activity was estimated by the formula

$$CB = V \times C \times A_1$$

where CB is the total cell-bound activity V is the olume of the phosphate buffer used for the autolytic release of the enzyme and A_1 is the optical density of the cell suspension at the start of the disintegration procedure and C is the specific enzyme activity

The amount of enzyme released was calculated by multiplying the volume of the culture sample supernatant with the enzyme activity per ml of the supernatant.

The amount of enzyme released into the wash buffer was calculated in the same way

The total enzyme activity of the culture sample was the sum of total cell-bound activity total activity released into the culture medium and total activity of the wash buffer

Temporary inhibition of autolysis. In order to investigate a possible surface location of hyaluronidase, which would permit extracellular activity of the cell-bound enzyme conditions had to be established under which the cells did not release hyaluronidase by autolysis. Washed cells were suspended in 0.1 M phosphate buffer at pH 6.8 and incubated t 37 C for 120 min. The cells remaining after this period were harvested by centrifugation, washed once in 0.1 M phosphate buffer and then resuspended in 0.01 M phosphate buffer pH 5.8 to an optical density of about $A_{540} = 24$ Cells of this suspension were resistant to autolysis at 37 C for at least 60 min as determined by optical density measurements and as judged from the absence of aminopeptidase activity in the supernatant.

Determination of selective release of hyaluronidase Selective release of hyaluronidase as an indication of a periplasmic location of the enzyme was investigated both with cells made resistant to autolysis by preincubation in 0 1 M phosphate buffer as described above and also with washed cells suspended in 0.01 M phosphate buffer at pH 6 8 without prior incubation. Release of hyaluronidase by sudden cooling of the cell suspension was tried according to *Neu & Heppel* (7) Release of the enzyme from the cells by vigorous treatment for 3–5 min of the cell suspension in a tissue grinder (A. H. Thomas, Philadelphia, USA) and release by osmotic shock according to *Heppel* (2) were also investigated.

Indicator of cell lysis. In the experiments carried out to investigate a surface location of hyaluronidase and selective release of the enzyme from undamaged cells, the release of aminopeptidase was used as an indication of cell lysis or cell damage. Previous investigations have shown that aminopeptidase may be used as a marker of intracellular material in experiments with *Streptococcus mitis* (3 5)

RESULTS

Specific and total hyaluronidase and aminopeptidase activities at different phases of the growth cycle From cultures growing under controlled conditions of anaerobiosis, pH, stirring and temperature, samples of 40

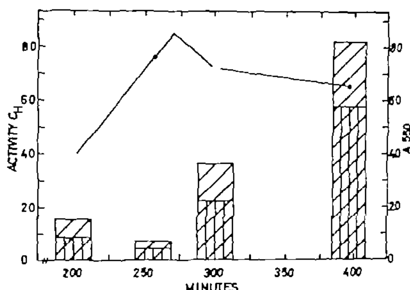


Fig 1 Specific hyaluronidase activity of cells from different growth phases The histograms are placed on the abcissa in such a way that the centres of histograms refer to the times of cultivation The disintegration of the cell samples was carried out both in phosphate buffer and in phosphate buffer supplemented with CAP Specific hyaluronidase activity of cells disintegrated in phosphate buffer [hatched] and in phosphate buffer supplemented with CAP [blank] Bacterial concentration at different growth phases ●—●

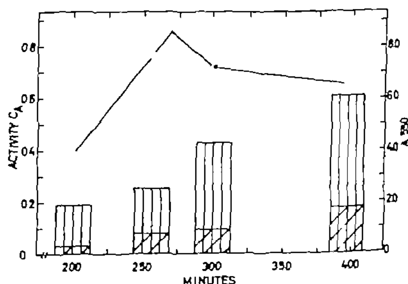


Fig 2 Specific aminopeptidase activity of cells from different growth phases The experimental data are presented as in Fig. 1 Specific aminopeptidase activity of cell disintegrated in phosphate buffer [hatched] specific aminopeptidase activity of cells disintegrated in phosphate buffer supplemented with CAP [blank] Bacterial concentration at different growth phases ● – ●

ml were taken 1) at the beginning of exponential growth, 2) at the end of exponential growth, 3) at the beginning of the autolytic phase about 30 *min after* the exhaustion of glucose, 4) *about 120 min* after the exhaustion of glucose

The disintegration of the cells by autolysis for release of cell-bound enzymes was carried

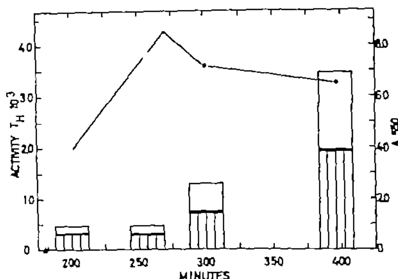


Fig. 3 Total hyaluronidase activity at different growth phases. Total cell-bound activity total activity occurring in the supernatant of the culture sample and total activity of the supernatant from the wash with buffer were calculated in samples of the culture. The histograms are placed on the abscissa in such way that the centres of the histograms refer to the times of cultivation. Total cell-bound activity [ ], total activity occurring in the sample supernatant [ ] and total activity of the supernatant from the wash with buffer [■]. Bacterial concentration at different growth phases ●—●

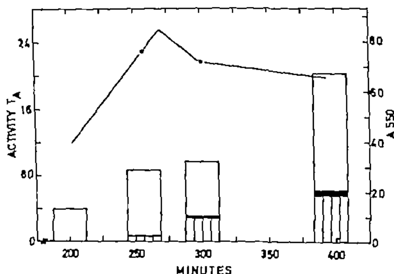


Fig. 4 Total aminopeptidase activity at different growth phases. The experimental data are presented as in Fig. 3. Total cell-bound activity [ ] total activity occurring in the supernatant of the culture sample [ ] and total activity of the supernatant from the wash with buffer [■]. Bacterial concentration at different growth phases ●—●

out both in buffer supplemented with CAP and in buffer without CAP. The autolytic process was not affected by CAP. As can be seen from Fig. 1 CAP reduced the yield of hyaluronidase in the extracts. This result indicated that hyaluronidase was synthesized during incubation in buffer in the absence of inhibitor of protein synthesis. On the other

hand, CAP increased the yield of aminopeptidase under these conditions (Fig 2) This result was in accordance with the observation in a previous investigation (3) The comparison between cell-bound and quantities of the released enzymes presented in Figs. 3 and 4 is based on data obtained from autolytic release of cell-bound hyaluronidase in the presence of CAP while the cell bound aminopeptidase activity was calculated after disintegration in the absence of CAP

The histogram in Fig 1 shows that the specific hyaluronidase activity decreased during exponential growth but increased rapidly *after the cessation of exponential growth.* The specific aminopeptidase activity increased *during the whole period of cultivation* (Fig. 2)

Both the cell-bound fraction and the released fraction of hyaluronidase of the culture remained constant during exponential growth (Fig 3) During the autolytic phase, both fractions increased so that the total hyaluronidase activity of the culture increased more than 7 fold from the end of the exponential growth phase to about two hours after the exhaustion of glucose.

The released fraction of hyaluronidase in the exponential growth phase corresponded to the amount of enzyme occurring in the extracellular medium of the precultures. No extracellular aminopeptidase activity was found in the precultures, most probably because of inactivation of the enzyme at the acidic pH (pH 5.2–5 4) of these cultures.

The amount of cell-bound aminopeptidase increased during the whole culture period. Increasing amounts of released aminopeptidase occurred in the autolytic phase. A small fraction of the total aminopeptidase, about 10 per cent, occurred in the extracellular medium at the end of exponential growth (Fig 4)

The amounts of both enzymes recovered in the supernatants from washes with buffer were negligible compared to the released and cell-bound quantities (Figs. 3 and 4)

Proneness to autolysis of cells of different phases of the growth cycle The autolytic capacity of cells of different growth phases could be directly compared since the cells of the four samples were suspended in phosphate buffer to the same optical density for the autolytic disintegration procedure. No significant difference in autolysis of cells harvested in early and late exponential growth phase was observed. The optical density of the cell suspensions decreased by about 45 per cent. Cells harvested in the autolytic phase showed decreasing ability to autolyse. However it was observed that autolysis in phosphate buffer was inversely proportional to the autolysis that had occurred *in the culture at the time of harvest* so that the total percentage of autolysis was the *same at all phases of growth. It may be* concluded that a constant fraction of exponentially growing cells are capable of *autolysis* under specified conditions.

Determination of hyaluronidase activity by intact cells Degradation of hyaluronate by intact cells without release of hyaluronidase from the cells would indicate a surface location of the enzyme. Hyaluronate, in a final concentration of 0.2 per cent (w/v) was added to a suspension of autolysis-resistant cells prepared as described. Incubation was then carried out at 37 C for periods ranging from 2 min to 30 min. The cells were then removed by centrifugation and the viscosity of the hyaluronate solution was determined viscosimetrically and compared with a control solution incubated without cells but otherwise treated like the test solutions. The hyaluronate solutions were also assayed for aminopeptidase activity as an indication of cell lysis. No reduction in the viscosity of the hyaluronate solutions could be observed after incubation for up to 15 min. Longer incubation periods resulted in disruption of the cells as determined by the aminopeptidase activity of the solutions, and also in a decrease in viscosity of the solution due to release of hyaluronidase.

Thus, intact cells did not seem to hydrolyse hyaluronic acid. The cells were less resistant to autolysis in hyaluronate solution than in buffer

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FORMATION AND RELEASE OF HYALURONIDASE AND AMINOPEPTIDASE IN NON-GROWING CELLS OF *STREPTOCOCCUS MITIS* ATCC 903

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Hyaluronidase synthesis in *Streptococcus mitis* (ATCC 903) was completely repressed during anaerobic growth in glucose-containing media. A rapid synthesis of hyaluronidase was observed when exponentially growing, repressed cells were transferred to a proteose-peptone medium lacking fermentable carbohydrates. The synthesis started after a lag of approximately 10 min and continued at a constant rate for about 20 min. Chloramphenicol completely inhibited the synthesis. The period of synthesis of hyaluronidase at maximal rate increased with increasing concentrations of proteose-peptone, while the rate of synthesis was not accelerated by proteose-peptone concentrations above 1 per cent. Aminopeptidase activity of the cells also increased in proteose-peptone medium. This increase was not inhibited by chloramphenicol or by puromycin. The aminopeptidase activity of the cells increased with increasing concentrations of proteose-peptone. The increase of both enzymes was most rapid at pH 7.4–7.7. The cells released hyaluronidase and aminopeptidase solely by autolysis. Autolysis in proteose-peptone medium was most rapid at pH 7.4–7.7 and was not affected by variations in the proteose-peptone concentration or by chloramphenicol.

In a previous article (6) it was shown that the synthesis of hyaluronidase in *Streptococcus mitis* (ATCC 903) was completely repressed by glucose during anaerobic growth. Synthesis of the enzyme took place during a period of autolysis which followed upon the exhaustion of glucose. Aminopeptidase activity of the cells, on the other hand, increased during exponential growth and during the period of autolysis following glucose depletion.

Regulation mechanisms of enzyme synthesis are usually studied in growing cultures or in resting cell suspensions. The term resting cell is generally applied to cell suspensions deficient in some exogenous nutrient, most often the nitrogen source, so that growth is prevented. The present investigation was performed by cells suspended in peptone media deficient in growth-promoting carbohydrates. As the cells in this system were far from resting, although no growth occurred, and in order to avoid confusion with nitrogen-deficient resting cells, the term non-growing cells was considered appropriate for the experimental system.

The aim of the present investigation was to study derepression of hyaluronidase synthe-

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ase by transferring repressed cells from a culture growing logarithmically in a glucose-proteose-peptone medium to carbohydrate-free proteose-peptone medium. The term derepression has been used by *Baumberg* (1) to indicate a rise in the rate of synthesis following the release of repression. The experimental system also offered the advantage of studying the effect of some factors on enzyme synthesis separated from the effect on growth and carbohydrate metabolism. Such factors affecting the synthesis of hyaluronidase included in this investigation were pH of the medium, concentration of peptones and the effect of inhibitors of protein synthesis.

In two previous articles on hyaluronidase activity of *Streptococcus mitis* (4 6) the aminopeptidase activity of the cells was studied in parallel. Aminopeptidase was of particular interest as an intracellular marker in these cells, but it was also considered valuable to compare variations in cell-bound and released enzyme activity of two different enzymes during the growth cycle. Aminopeptidase activity was included in the present investigation in order to continue the comparison between the two enzymes. Since autolysis was the sole mechanism for the physiological release of hyaluronidase and aminopeptidase conditions affecting the autolytic process were also studied.

TABLE 1 *Experimental Procedure*

Part	Step	Procedure
I	1	Cultivation in fermentor at controlled pH (6.5) Medium PP I (containing 1 per cent glucose w/v)
	2.	Culture samples of 40 ml withdrawn at the end of the logarithmic growth phase or in the autolytic phase about 15 min after glucose exhaustion.
	3	Centrifugation. Supernatants discarded
	4	Washing of cells in cold 0.01 M phosphate buffer pH 6.8.
	5	Centrifugation. Supernatants discarded
II	6.	Resuspension in carbohydrate-free media.
	7	Homogenization of cell suspensions.
	8.	Incubation of cell suspensions at 37° C.
	9	Centrifugation. Supernatants taken for assay of enzyme activity
III	10	Resuspension of cells in 0.05 M phosphate buffer pH 6.8.
	11	Homogenization of cell suspensions
	12.	Immediate determination of optical density of diluted samples of cell suspensions.
	13	Incubation for 20 hours at 37 C.
	14	Homogenization of cell suspensions.
	15	Immediate determination of optical density of diluted samples of cell suspensions
	16.	Centrifugation. Supernatants taken for assay of enzyme activity. Pellets discarded.
IV	17	Determination of enzymatic activity of supernatants obtained at steps 9 and 16

MATERIALS AND METHODS

Strain. *Streptococcus mitis* ATCC 903

Experimental procedure. The experimental procedure is summarized in Table 1. The procedure consisted of four major parts (I IV). In part I equal cell samples were withdrawn from a population of cells of a defined physiological state and washed once. *In part II the washed cell samples* were suspended in experimental media and incubated in centrifugal tubes with tight caps at 37 C. The cell suspensions had a cell density corresponding to approximately 4 mg dry weight of cells per ml. Part III of the procedure concerned the extraction of the enzymes from the cells by autolysis. Part IV concerned the determination of enzymic activities of the cell-free extracts of the cell samples.

It will be shown in this article that cells of the logarithmic growth phase, repressed in hyaluronidase synthesis, differed from cells of the autolytic phase of growth regarding the kinetics of hyaluronidase synthesis. In the present investigation the cells were harvested in the late logarithmic growth phase about 20 min before the exhaustion of glucose and also at the beginning of the autolytic phase about 15 min after the exhaustion of glucose. The observation made in a previous study (4) that the consumption of titrant used to keep pH constant during growth was parallel to growth and glucose consumption, permitted control of the growth cycle of the culture and made harvest of cells at fixed stages of the growth curve possible.

Media and cultivation techniques. The cultivation according to step 1 in the experimental procedure was performed at controlled pH and under anaerobic conditions in a stirred culture vessel (FG 500, Biotec, Stockholm, Sweden). The cultivation technique and the medium used, called PP I have been described in a previous paper (4).

The media used for incubation of cells under non-growing conditions (steps 6–8) were the following. A proteose-peptone medium pH 6.9, called

PP 2 of the following composition Proteose-peptone (Difco) 40 g, $Na_2HPO_4 \cdot 2H_2O$ 7.5 g, KH_2PO_4 3.0 g, Ca-panthothenate 1.2 mg, nicotinic acid 1.2 mg, pyridoxine-HCl 1.2 mg, thiamine-HCl 1.2 mg, pimelic acid 1.2 mg, riboflavine 0.12 mg, $MgSO_4 \cdot 7H_2O$ 20 mg, $MnSO_4 \cdot 4H_2O$ 10 mg, $FeSO_4 \cdot 7H_2O$ 6 mg, citric acid 6 mg Distilled water was added to a final volume of 1 litre. The pH of the PP 2 medium was raised to pH 7.4 by increasing the amount of Na_2HPO_4 to 10.5 g per litre and omitting KH_2PO_4. This medium was called PP 3.

In some experiments, the proteose-peptone part of the PP 3 medium was exchanged for the same amount of Trypticase, a tryptic digest of casein (B D Merieux, Marcy L'étoile, Rhône, France)

Sterilization of the PP 2, PP 3 and trypticase media was performed by filtration. The membrane filter had a pore size of 0.22±0.02 µm (Millipore Filter Corp., Bedford, Mass., USA) The media were passed through the filters by positive pressure which did not exceed 50 p.s.i. Centrifugations were performed at 20,000 × g for 10 min at 4 C. Homogenization of the cell suspensions was performed with a tissue grinder (A. H. Thomas, Philadelphia, Pennsylvania, USA)

Enzyme assays. Hyaluronidase activity was assayed by the viscosimetric method described by *Hultin* (3) The activity was calculated by the formula of *Hultin* (3) and expressed in Hultin units (H.U.) per ml. Aminopeptidase activity was determined by modification of the method for determination *of leucine aminopeptidase activity* described by *Goldberg & Rutenburg* (2) The modified method was used in a study on aminopeptidase activity in cell-free extracts of *Streptococcus mitis* (7) Aminopeptidase activity was expressed in units (U) per ml.

Materials. Hyaluronic acid grade III-S was obtained from Sigma Chemical Co., St. Louis, Mo., USA. Reagents for the determination of aminopeptidase activity and puromycin were also obtained from Sigma Chloramphenicol (CAP) was purchased from Park-Davies.

Extraction of the enzymes. Both hyaluronidase and aminopeptidase have been shown to be cell-bound enzymes in *Streptococcus mitis* and to be released by disruption of the cells (6) In the present investigation, the extraction of both enzymes was performed by autolysis carried out in 0.05 M phosphate buffer pH 6.8 containing chloramphenicol (CAP) 100 µg/ml for 20 hours at 37 C. The extraction of the enzymes by this procedure has been described previously (5)

Calculations. The specific enzyme activity as an expression of the enzymic content of the cells was calculated by the formula

$$C = \frac{E}{\Delta OD}$$

where C is the specific enzyme activity (C_H specific hyaluronidase activity C_A specific aminopeptidase activity) E is enzyme activity per ml of cell-free extract, ΔOD is the measurement of cellular disintegration obtained by the difference in optical density of the cell suspension at the beginning of the incubation in buffer and at the end of the incubation period, as described previously (5)

Total enzyme activity was calculated by the formula

$$T = v \times E_m + V \times A_1 \times C$$

where T is total activity; v is volume of the experimental medium E_m is enzyme activity per ml of the medium V is volume of the phosphate buffer A_1 is the optical density of the cell suspension at the start of autolysis in the buffer and C is the specific enzyme activity

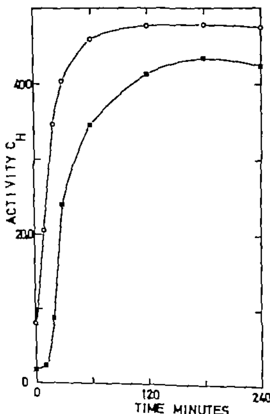


Fig. 1. Kinetics of derepression of hyaluronidase synthesis. Glucose repressed cells and cells relieved of glucose repression were harvested from the same culture at different phases of the growth cycle and incubated in proteose-peptone *medium. The* specific hyaluronidase activity (C_H) is plotted against incubation time. Glucose repressed cells ■——■ cells relieved of glucose repression ○——○.

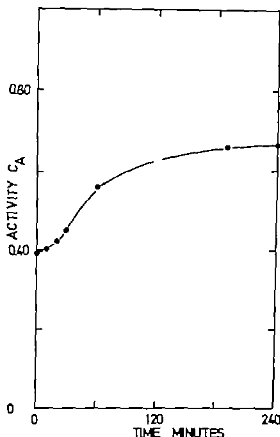


Fig 2 Kinetics of the increase in aminopeptidase activity of the cells during incubation in PP 3 medium. The specific aminopeptidase activity (C_A) was determined after incubation of cells at 37 C for various periods of time.

RESULTS

Kinetics of Enzyme Formation

In order to study the kinetics of hyaluronidase synthesis in derepressed cells, the cells were transferred from a medium containing glucose to a medium lacking glucose and other fermentable carbohydrates. Cell samples were harvested from the late logarithmic phase of a culture and suspended in equal volumes of PP 3 medium. Incubation of the cell suspensions was carried out for various periods of time. The hyaluronidase activity of the cells is plotted against incubation time in Fig 1 It is indicated by this figure that the synthesis of the enzyme started after a lag of about 10 min and proceeded at maximal rate for about 20 min. The rate of synthesis then declined and no increase in the specific hyaluronidase activity was observed after 120 min of incubation.

Experiments were also carried out to investigate whether the lag in hyaluronidase synthesis reflected the time necessary for derepression or it was merely an expression of a delayed protein synthesis caused by the washing and transfer procedures. Cells relieved of glucose repression were harvested from the autolytic phase of the fermentor culture about 15 min after the exhaustion of glucose and transferred to PP 3 medium. The synthesis of hyaluronidase in these cells started without any detectable lag (Fig 1)

At the time when cells harvested in the late logarithmic phase were transferred to PP 3 medium, the hyaluronidase content of the cells was low due to glucose repression during

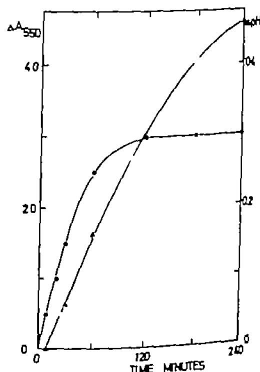


Fig 3 Autolysis and decrease in pH in PP 3 medium during incubation of cells at 37° C. Autolysis, determined as the loss in optical density (ΔA_{550}) of cell suspensions having an initial optical density of A_{550} = 1 10 ± 0 1 and the decrease in pH (ΔpH) are plotted against incubation time ΔA_{550} ▲—▲, ΔpH ●—●

growth, while the specific aminopeptidase activity had increased during the preceeding cultivation (6) During the subsequent incubation in PP 3 medium the specific aminopeptidase activity increased (Fig. 2) The percental increase in aminopeptidase activity of the cells was considerably less than the increase in hyaluronidase activity and the shape of the plot of specific aminopeptidase activity versus incubation time (Fig. 2) was sigmoid.

Autolysis during Incubation of Non-growing Cells

It has been shown earlier (4) that *Streptococcus mitis* cells autolyze when deprived of growth promoting carbohydrates. Thus, autolysis occurred also during the incubation in carbohydrate-free PP 2 and PP 3 media. Lysis caused the release of acidic cell products which decreased pH of the media. The decrease in optical density during incubation in PP 3 medium and the decrease in pH of the medium is plotted against time in Fig. 3 The initial optical density decreased by about 35 per cent during incubation of cells in PP 3 medium for 240 min. For comparison it is worth pointing out that cell lysis in 0.05 M phosphate buffer was about 27 per cent at pH 6.8 in 240 min at 37 C (5)

The Effect of Inhibitors of Protein Synthesis on the Formation of Hyaluronidase and Aminopeptidase in Non growing Cells

Studies of different sugar degrading enzyme systems, which have been performed in strains of *Escherichia coli* have all shown that regulation of these enzymes is performed entirely by control of enzyme synthesis rather than by control of activity (1) The following experiments were performed in order to investigate the effect of inhibitors of protein synthesis, chloramphenicol (CAP) and puromycin, on the observed increase in hyaluronidase and aminopeptidase activities in non-growing cells. Cells were suspended in PP 3 media containing CAP at final concentrations ranging from 100 to 400 µg/ml and incubated at 37 C for 60 min. Cells were also incubated in PP 3 medium without CAP as control. No increase in the specific hyaluronidase activity occurred in the presence of CAP The specific aminopeptidase activity however increased in the CAP-media to the same extent as in the control. The release of aminopeptidase into the PP 3 – CAP media increased with increasing amounts of CAP in the medium. This phenomenon was similar to that observed by incubation of cells in phosphate buffer containing various concentrations of CAP which has been described earlier (5)

Puromycin added to PP 3 medium at a concentration of 500 µg/ml inhibited completely the synthesis of hyaluronidase while the aminopeptidase activity of the cells increased to the same extent as in the control medium with no puromycin. The specific effect of CAP on the release of aminopeptidase from autolyzing cells was not found in experiments with puromycin. The results indicated that the increased hyaluronidase activity of the non-growing cells reflected an increased rate of synthesis, while the increase in aminopeptidase activity of the cells was not regulated by control of synthesis.

Effect of CAP upon Autolysis

Autolysis in the PP 3 – CAP media was the same as in the control. Incubation in PP 3 – CAP media did neither affect autolysis during the subsequent incubation in phosphate buffer

Effect of pH upon the Formation of Hyaluronidase and Aminopeptidase and upon Autolysis

Samples of logarithmically growing cells were suspended in PP 2 media of various pH. The pH of the media were set by sterile NaOH and HCl. The cells were incubated for 60 min at 37 C.

As pointed out earlier in this article, autolysis occurred during incubation in PP 3 and PP 2 media which caused a decrease in pH of the media. The decrease in pH in the media of various pH was in the range 0 1–0.4

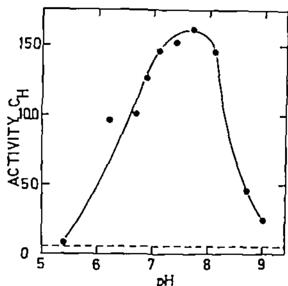


Fig 4 Effect of pH on the synthesis of hyaluronidase in non-growing cells. The specific hyaluronidase activity (C_H) determined after incubation of *cells in PP 2 media of various pH for 60 min at* 37 C is plotted against pH of the medium. The stippled line indicates the specific hyaluronidase activity at zero time.

units and was dependent on the degree of *autolysis and on the* buffer capacity of the media. In Figs. 4 and 5 are shown the specific hyaluronidase and aminopeptidase activities plotted against pH of the media which were calculated as the mean value of pH before and after the incubation of cells. The rate of increase in specific enzyme activities was significantly affected by pH of the medium. The synthesis of hyaluronidase was *most rapid in cells incubated at pH 7 7* The specific aminopeptidase activity increased most rapidly during incubation at pH 7 4 Autolysis, and consequently the release of the enzymes during incubation in PP 2 media, was also affected by pH. As can be seen from Fig 6, autolysis was most rapid at pH 7 4–7 7 *It was also* observed that lysis during the subsequent incubation in phosphate buffer was inversely proportional *to lysis in PP 2* media. The sum of decrease in optical density in PP 2 medium and in buffer was approximately the same for cells incubated in PP 2 media in the pH range 6.2–9 0 For cells incubated at pH 5 4 the total decrease in optical density was about 25 per cent less.

When studies in repression of hyaluronidase synthesis are performed in growing cultures under conditions where pH is not controlled it is *of interest to know at what acidic pH* hyaluronidase synthesis is completely inhibited and how pH affects the activity of cell-bound hyaluronidase. Therefore the following experiments were carried out. Cells relieved of glucose repression were sampled 15 min after glucose exhaustion and transferred to *PP 2 media of low pH* The pH of the media ranging from 5 7 to 4.5 had been set by sterile *HCl.* The cells were *incubated at* 37 C for 60 min. Cells were also incubated in PP 2 medium (pH 6.9) supplemented with CAP (100 μg/ml) as control. From the results obtained which are shown in Table 2, it may be concluded that the rate of increase in total hyaluronidase activity declined with decreasing pH. *At pH 4.9* the total activity decreased by about 4 per cent compared to the control and at pH 4.5 a significant decrease in total activity was observed. As autolysis and consequently the release of the en-

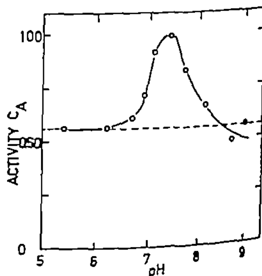


Fig 5 Effect of pH on the increase in aminopeptidase activity of cells incubated in PP 2 media of various pH for 60 min at 37 C. The specific aminopeptidase activity is plotted against pH of the medium. The stippled line indicates the specific aminopeptidase activity at zero time

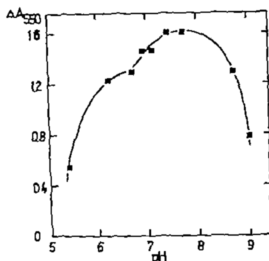


Fig 6 Effect of pH on autolysis in PP 2 media. The cells were incubated in PP 2 media of various pH for 60 min at 37 C. The initial optical density of the suspensions was $A_{550} = 12.0 \pm 0.1$ Autolysis, expressed as the decrease in optical density (ΔA_{550}) is plotted against pH of the medium

zyme decreased with decreasing pH it may be concluded that the main loss in activity was due to inactivation of the enzyme at the cell bound site.

Effect of Peptones upon Formation of Hyaluronidase and Aminopeptidase

It has been shown earlier that proteose-peptone was essential for synthesis of hyaluronidase and that a tryptic digest of casein could not replace proteose-peptone in this respect (4) In the present investigation, the effect of various concentrations of proteose peptone on the formation of hyaluronidase and aminopeptidase was studied in non-growing cells. Samples of cells were incubated in PP 3 media of different concentrations of proteose peptone for various lengths of time at 37 C. In Fig 7 the specific hyaluronidase activity is plotted against time. The initial rate of synthesis in 8 per cent, 4 per cent and 1 per cent proteose-peptone did not vary significantly Synthesis in these media occurred at constant rate for periods of time increasing with the proteose peptone concentration. The synthesis in 4 per cent and 1 per cent proteose-peptone stopped within 240 min, while the synthesis in 8 per cent proteose-peptone showed no sign of decline at the end of the experimental period. The specific hyaluronidase activity also increased in 0.1 per cent proteose peptone. In experiments where the proteose-peptone part of the PP 3 medium was replaced by Trypticase, 4 per cent (w/v) only a very small increase in specific hyaluronidase activity could be observed after 240 min of incubation (Fig 7)

The rate of formation of active aminopeptidase increased with increasing proteose peptone concentrations of the medium as can be seen from Fig 8 The aminopeptidase con-

TABLE 2. *The Effect of Low pH on Cell-bound and Total Hyaluronidase Activity*

Medium	pH	Activity occurring in the medium H U	Cell-bound activity H.U	Total activity H.U
PP 2	5 7	823	3952	4775
PP 2	5.3	457	2396	2853
PP 2	5.1	256	2418	2673
PP 2	5 0	196	1748	1944
PP 2	4.9	60	1730	1790
PP 2	4.5	48	436	484
PP 2 + CAP	6.9	117	1744	1861

Cells relieved of glucose repression were incubated in PP 2 media of various acidic pH. The incubation was carried out for 60 min at 37 C. Cells were also incubated in PP 2 medium containing CAP (100 µg/ml) as control. The total hyaluronidase activity i.e. the sum of the activity occurring in the medium and the activity bound to the cells at the end of the incubation period was calculated.

seems a more likely mechanism. However differences in the sensitivity of the synthesis of certain proteins to CAP have been reported by *Vambutas & Salton* (12) who found that the synthesis of Ca^{++}-dependent adenosine triphosphatase of *Micrococcus lysodeikticus* was resistant to CAP while the synthesis of certain cytoplasmic enzymes was inhibited. Puromycin inhibited the synthesis of both ATPase and the cytoplasmic enzymes. It was pointed out by these authors that the inhibitory effect of CAP on the synthesis of certain membrane proteins varied between bacterial species. An interesting hypothesis has been advanced by *Simmonds & Toye* (10) stating that peptidases in bacteria exist in a partially latent form and that the activity is controlled by environmental factors among which the metal ion content and pH are the most important ones. The finding in the present investigation that the specific aminopeptidase activity increased with increasing proteose-peptone concentration of the medium during incubation might be explained in terms of increasing concentrations of certain metal ions. In fact, partially purified aminopeptidase of *Streptococcus mitis* was found to be activated by Co^{++} (unpublished results).

Since hyaluronidase synthesis in growing and non-growing cells could not be increased by the addition of hyaluronic acid nor by degradation products of hyaluronic acid (unpublished data) hyaluronidase must be considered a constitutive enzyme in this strain of *Streptococcus mitis*. The parallelism between the kinetics of derepression of constitutive synthesis and the kinetics of induction of induced enzyme synthesis is obvious. In most inducible systems the synthesis starts, if enough inducer is added, after a short lag of usually a few min or less. In the present investigation, depressed constitutive hyaluronidase synthesis started after a lag of about 10 min while the synthesis in unrepressed cells, harvested in the autolytic phase of the culture continued without any detectable lag if the cells were transferred to proteose-peptone medium. Thus, the observed lag in hyaluronidase synthesis may reflect the time both for lowering the concentration of effectors of repression and for synthesis of measurable amounts of active protein.

The initial rate of hyaluronidase synthesis did not seem to be significantly increased by proteose-peptone concentrations above 1 per cent, while the level of hyaluronidase in the cell obtained in 4 hours was dependent on the concentration of proteose-peptone. In a system as that in the present investigation, the lack of sources of energy is most probably a limiting factor in enzyme synthesis. It may be speculated that the amino acids, the building blocks of the enzymes, were derived from peptides transported across the cytoplasmic membrane mainly by diffusion or facilitated diffusion and not by energy-consuming active transport of amino acids or peptides. The diffusion being limited by the extracellular concentration of peptides of suitable size and structure. The intracellular peptidase activity would facilitate the diffusion by rapid cleavage of peptides to amino acids (8).

It was observed in a previous study (4) that hyaluronidase activity of cultures of *Streptococcus mitis* was increased in media containing proteose-peptone but that a casein hydrolysate could not stimulate hyaluronidase activity of the cultures. These observations were confirmed in the present investigation of non-growing cells. The failure of the casein hydrolysate to stimulate hyaluronidase synthesis may be due to insufficient concentrations of peptides of specific size and structure necessary for diffusion into the cells or shortage of some amino acids essential for protein synthesis in non-growing cells of *Streptococcus mitis*.

It has been shown previously (6) that the release of hyaluronidase takes place solely by disruption of the cells. Therefore the autolytic process of this strain has attracted a special interest as a release mechanism for the two cell-bound enzymes. In a work on autolysis of *Streptococcus faecalis Shockman et al.* (11) have suggested that the autolytic process has two distinct aspects (1) the conditions that lead to the formation of cells

that are prone to autolysis and (2) those that allow the expression of the lytic capacity of the cells. The expression of the lytic capacity of *Streptococcus mitis* in phosphate buffer was affected by salt concentration, pH and by temperature (5) Autolysis in proteose-peptone medium was maximal at the same pH-range (7.4–7.7) as in phosphate buffer as shown in the present investigation.

The experimental data of the present work show that chloramphenicol added to non-growing cells at concentrations sufficient to inhibit hyaluronidase synthesis could neither affect the expression of the autolytic capacity nor the capacity to autolyze. This finding is in contrast to that obtained by *Sayare et al.* (9) who found that chloramphenicol and other inhibitors of protein synthesis rapidly decrease the ability of cells of *Streptococcus faecalis* to autolyze. The observation that cells incubated in PP 2 medium at pH 5.6 showed less total autolysis may indicate that low pH irreversibly decreased the capacity of the cells to autolyze.

Experimental investigations of protein synthesis have frequently been performed by studies of induced enzyme formation in bacteria and yeasts. Reports on constitutive enzyme synthesis, however have been comparatively rare. Some of the reasons for this have been the difficulties involved in a separation of the effects on growth from the effects on enzyme synthesis of the factors under study. The great variety of changes accompanying growth have also complicated the interpretation of the results. The experimental procedure employed in the present investigation has overcome some of these difficulties. The reproducibility of the experiments increased by the use of cells in a defined physiological condition.

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REPRESSION OF HYALURONIDASE IN *STREPTOCOCCUS MITIS*, ATCC 903

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The synthesis of hyaluronidase, which is a constitutive, cell-bound enzyme in *Streptococcus mitis* ATCC 903 was investigated in cultures grown on different carbohydrates. Mannose was found to repress enzyme synthesis completely. The repressive effect of glucose was concentration dependent. After a period of complete repression for less than one generation, the rate of synthesis increased rapidly in 0.06 M glucose while complete repression continued in 0.17 M glucose. Sucrose, fructose and galactose repressed enzyme synthesis only to a limited extent. Of all carbohydrates tested sucrose had the least repressive effect and supported a maximal rate of growth. Cyclic AMP partially relieved glucose repression, particularly in media of low glucose concentration. The same effect was, however also obtained with 5 AMP. Theophylline did not increase the effect of cyclic AMP in counteracting glucose repression. Cyclic AMP caused 30–50 per cent inhibition of the synthesis of hyaluronidase in non-growing cells.

The repressive effect on the rate of synthesis of certain enzymes exerted by glucose or any compound which can serve as an efficient source of metabolites and of energy was termed catabolite repression by *Magasanik* (7). The extensive literature on catabolite repression has been reviewed by *Paigen & Williams* (11). In almost all examples of catabolite repression glucose was effective. The repressive effect of glucose on the synthesis of hyaluronidase in cells of *Streptococcus mitis* strain ATCC 903 has been reported previously (5). It was observed that, under anaerobic conditions, glucose inhibited the synthesis of hyaluronidase and that the inhibition was not relieved until glucose was exhausted. *Paigen & Williams* (11) concluded that carbon sources which supported a rapid rate of growth were the most effective in catabolite repression. However it was not generally true that the intensity of catabolite repression was proportional to growth rate in all media. Conflicting results have been reported on the effect of low temperatures on catabolite repression of β-galactosidase in strains of *Escherichia coli* (9, 10).

Pastan & Perlman (12) postulated that catabolite repression in bacteria might be due to the lowering of the intracellular concentration of the cyclic nucleotide adenosine 3′ 5′-monophosphate (cyclic AMP). It has been shown in several reports that the addition of cyclic AMP counteracted glucose repression of enzyme synthesis in many gram-negative micro-organisms (12). *Aboud & Burger* (1) reported that cyclic AMP-phosphodiesterase affected the efficiency of exogenous cyclic AMP in relieving catabolite repression of β-galactosidase in *E. coli* and that cyclic AMP-phosphodiesterase was in-

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hibited by theophylline. Theophylline almost doubled the efficiency of the exogenous cyclic AMP in stimulating the differential rate of β-galactosidase synthesis.

The present study was designed to investigate (1) the differences between some carbohydrates as repressors of hyaluronidase synthesis and their effect on growth rate, (2) the influence on repression of lowering the temperature, (3) the ability of exogenous cyclic AMP to counteract the repression of hyaluronidase synthesis and (4) the effect of cyclic AMP on the synthesis of hyaluronidase in non-growing cells during incubation in carbohydrate free medium. Furthermore, since the addition of cyclic AMP was found to relieve only partially repression, it was considered worthwhile to investigate the effect of theophylline on the ability of cyclic AMP to relieve repression of hyaluronidase synthesis in this strain.

MATERIALS AND METHODS

Bacterial Strain. Streptococcus mitis, *ATCC 903*

Experimental procedure The experimental procedure is summarized in Table 1 The cell samples in each experiment were withdrawn at the same time from a culture growing logarithmically under controlled conditions of anaerobiosis, pH, stirring and temperature. The nutrient medium (PP1) and the cultivation technique have been described earlier (3) Centrifugations were performed at 20 000 $\times g$ for 10 min at 4 C.

Experimental media. The experimental media (step 6) were the PP2 and PP3 media previously described (6) Sugars, nucleotides, theophylline and chloramphenicol (CAP) were added to media prior to sterilization. Sterilization was performed by filtration through a membrane filter having a pore size of 0.22 ± 0.02 μm (Millipore Filter Corp., Bedford, Mass USA)

In all experiments, one cell sample was incubated in PP2 or PP3 medium containing CAP (100 μg/ml) and another in PP2 or PP3 medium without added sugar as control of enzyme synthesis by non-growing cells, as described earlier (6)

Experimental conditions The cell suspensions were incubated in centrifuge tubes (50 ml) with tight caps (step 8) For practical reasons, pH of the cultures could not be controlled and thus decreased during growth.

Enzyme assay Hyaluronidase activity was assayed as previously described (3)

TABLE 1 *Experimental Procedure*

1 Cultivation in fermentor Medium PP1 (containing glucose 1 per cent w/v)
2 Culture samples of 10, 20 or 40 ml withdrawn at the end of logarithmic growth phase.
3 Centrifugation. Supernatants discarded.
4 Washing of cells in cold 0.01 M phosphate buffer pH 6.8.
5 Centrifugation. Supernatants discarded.
6. Resuspension of cells in the experimental media.
7 Homogenization of cell suspensions.
8. Incubation. The incubation time and temperature varied.
9 Centrifugation. Supernatants taken for assay of enzyme activity
10. Resuspension of cells in 0.05 M phosphate buffer pH 6.8.
11 Homogenization of cell suspensions.
12. Immediate determination of optical density of diluted samples of the cell suspensions.
13 Incubation for 20 hours at 37 C.
14 Homogenization.
15 Determination of optical density of diluted samples of cell suspensions.
16. Centrifugation. Supernatants taken for assay of enzyme activity Pellets discarded.
17 Determination of enzymatic activity of supernatants obtained at 9 and 16.

Extraction of hyaluronidase The extraction of cell-bound hyaluronidase from the cells was performed by autolytic disintegration of the cells as described earlier (4) Homogenization of the cell suspensions (steps 7 11 and 14 of Table 1) was made with the aid of a tissue grinder (A. H. Thomas, Philadelphia, USA)

Calculations The specific and the total hyaluronidase activities were determined by the formula given in a previous article (5)

Determination of bacterial growth Bacterial growth was determined by measuring the optical density at 550 nm of suitably diluted samples of cell suspensions in a Zeiss PMQ II spectrophotometer Relative growth was used as a measure of physiological time and expressed as A_t/A_0 where A_t and A_0 are the absorbancies at 550 nm at times t and 0.

Determination of glucose concentration. Glucose was assayed enzymatically by glucose-oxidase (Glox, AB Kabi, Stockholm, Sweden)

Chemicals. Adenosine 3' 5-monophosphate (cyclic AMP) was purchased from Sigma Chemical Co., St Louis, Mo. USA and from Boehringer Mannheim GmbH, Germany Adenosine 5-monophosphate, hyaluronic acid grade III-S and theophylline were obtained from Sigma and chloramphenicol from Park Davies.

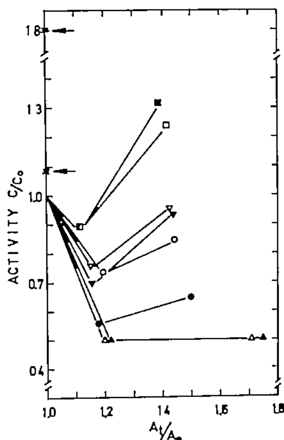


Fig 3 Effect of carbohydrates on repression of hyaluronidase synthesis during incubation at 7 C. Glucose-grown cells were incubated in aliquots in PP3 media containing various carbohydrates at concentrations of 0.06 M or 0 17 M. Incubation times 2 h and 20 h. Hyaluronidase activity is expressed as the specific activity (C) relative to the specific activity (C_0) of CAP-cultures.
△——△ mannose 0 06 M, ▲——▲ mannose 0.17 M, ○——○ glucose 0.06 M ●——● glucose 0.17 M ▽——▽ sucrose 0.06 M ▼——▼ sucrose 0 17 M, □——□ fructose 0.06 M ■——■ fructose 0.17 M.
The activity of cultures incubated in carbohydrate-free PP3 medium is indicated on the ordinate by arrows. The lower value represents the activity after incubation for 2 h and the upper value the activity after 20 h at 7 C.

dase activity of non-growing cells incubated in carbohydrate-free medium was included as control of synthesis. The specific and total activity in these cultures relative to CAP cultures are plotted along the ordinates in Figs. 1 and 2. The lower symbol represents the activity after incubation for 30 min and the upper symbol the activity after 60 min incubation at 37 C.

Growth rate is considered to be correlated to the phenomenon of catabolite repression (11) Information on growth rates in the different carbohydrate media is indirectly shown in Figs. 1 and 2. Symbols plotted nearer the ordinate for each carbohydrate represent relative growth in cultures incubated for 30 min and those further from the ordinate represent the same variable in cultures incubated for 60 min. Growth rate varied significantly in the different carbohydrate media. Sucrose and glucose supported the most rapid rate of growth. The enzymic content of glucose and mannose-cultivated cells had decreased by about 50 per cent after 30 and 60 min incubation (Fig 1) Sucrose- fructose- and galactose-cultivated cells increased their specific activity during the latter half of the 60 min incubation period. The enzymic level in sucrose-cultured cells increased during this period more rapidly than that of non-growing cells incubated in carbohydrate-free medium (Fig. 1)

The estimate of the total hyaluronidase activity takes into account the fraction of the enzyme released into the growth medium and also the effects of bacterial growth on the total amount of enzyme formed. From Fig. 2 it can be seen that the total activity in mannose-cultures after 30 min and 60 min and in glucose cultures after 30 min was less than the initial total activity It can also be seen from Fig 2 that sucrose increased the total activity 1.3 and 3 8 times in 30 min and 60 min fructose, 1 2 and 2.4 times and galactose, 1 1 and 1.2 times. The sucrose culture that was incubated for 60 min yielded higher total hyaluronidase activity than the culture incubated for 60 min in carbohydrate-free medium.

From these results it seems that glucose and mannose completely inhibited the synthesis of hyaluronidase while sucrose fructose and galactose inhibited the synthesis only to a limited extent. The reduction in the rate of enzyme synthesis in sucrose fructose and galac-

tive media was most pronounced during the first 30 min of incubation. Carbohydrates which did not support growth of this strain under the experimental conditions used did not affected the rate of synthesis of hyaluronidase as compared with the control. The following non-metabolizable carbohydrates were included in this study arabinose rhamnose, ribose and fucose.

Effect of Various Carbohydrates on Enzyme Synthesis at 7° C

As cultivation at low temperature decreased growth rate considerably experiments could be carried out for longer periods without exhaustion of the energy source. It was found advantageous to study the effects on hyaluronidase synthesis of two different concentrations of various carbohydrates at a low temperature.

Cells were incubated in PP3 medium containing 0.06 M or 0 17 M carbohydrate. Incubation was performed at 7 C for 2 and 20 h. The effect of different carbohydrates on the specific hyaluronidase activity of the cells is shown in Fig 3 Mannose supported the most rapid rate of growth and growth in mannose media decreased the enzymic content of the cells to the lowest level. The effect of glucose on the specific activity of the cells was concentration dependent. The high concentration of glucose decreased mostly the enzymic content of the cells. Fructose had the least repressive effect of hyaluronidase synthesis. By contrast to glucose, the effects of mannose, sucrose and fructose on the specific activity were not significantly affected when different concentrations of the carbohydrate were used. The specific activity of cells incubated in carbohydrate-free medium showed that synthesis of the enzyme occurred at 7 C under non-growing conditions.

Effect of Different Concentrations of Glucose on Enzyme Synthesis

The experiments performed at 7 C indicated that the repression of hyaluronidase synthesis by glucose was affected by concentration of glucose in the medium. Experiments were performed to compare the synthesis of hyaluronidase during growth at 37 C in PP3 media containing 0 06 M or 0 17 M glucose. The experimental data are presented in Fig. 4 and also the theoretical curve calculated on the assumption that synthesis of hyaluronidase was completely inhibited and that the existing enzyme was diluted out by growth. Since the synthesis of hyaluronidase in non-growing cells (6) on which interpretation of these results depends,

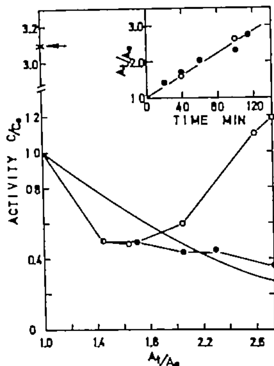


Fig 4 Effect of glucose concentration on repression of hyaluronidase synthesis. Cells were incubated in PP3 media containing glucose at a concentration of 0.06 M or 0 17 M. Incubation times varied between 20 to 110 minutes. Incubation temperature was 37 C. Hyaluronidase activity is expressed as the specific activity (C) relative to the specific activity (C_0) of the CAP-culture. Growth is expressed as the relative growth (A_t/A_0)
O——O cultures grown in PP3 medium containing 0.06 M glucose, ●——● cultures grown in PP3 medium containing 0.17 M glucose.
The continuous curve is the theoretical curve representing total repression of enzyme synthesis. Relative growth (A_t/A_0) is plotted against time in insert.

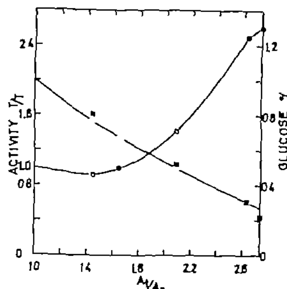


Fig 5 Repression of hyaluronidase synthesis. Cells were grown in PP3 medium containing glucose (0 06 M) (the same experiment as in Fig. 4) The concentration of glucose remaining in the extra cellular medium is plotted against relative growth. Hyaluronidase activity is expressed as total activity (T) relative to the total activity (T_0) of the CAP culture. Growth is expressed as relative growth (A_t/A_0)
○——○ total activity (T/T_0) ■——■ residual glucose concentration of the external medium.

was decreased at low pH and completely inhibited at pH below 5 1 the present experiments were terminated at pH 5 1

No difference in growth rate was observed in media of high and low glucose concentrations (Fig 4 insert) and the decrease in pH in the two series of cultures occurred at the same rate. The specific activity of cells grown at low glucose concentration was below the theoretical curve for about one generation thereafter the specific activity increased rapidly during growth. At high glucose concentration, the specific activity was below the theoretical curve for a longer physiological time and reached values only slightly above that curve at the end of the experiment (Fig. 4) It appears from Fig 5 that the total activity of low glucose cultures increased to values above the initial within the first generation when the residual glucose concentration of these cultures was about 0.5 per cent.

Effect of Exogenous Cyclic AMP on the Synthesis of Hyaluronidase in Growing and in Non growing Cells

The effect of various concentrations of cyclic AMP in the medium on growth and on repression of hyaluronidase synthesis was studied using growing cells for 90 min at 37 C in PP3 media containing 0.17 M glucose and 1–12 mM cyclic AMP Three controls were included in these experiments control for synthesis in carbohydrate-free PP3 medium, control for synthesis in carbohydrate-free PP3 medium + 8 mM cyclic AMP control for repression in PP3 medium containing 0 17 M glucose.

The experimental data, presented in Table 2, suggest that cyclic AMP partially relieved the pronounced repression exerted by 0.17 M glucose. It appears also from Table 2 that

TABLE 2 *Effect of Cyclic AMP on the Synthesis of Hyaluronidase*

Medium	Bacterial concentration relative to the initial (A_t/A_0)	Specific activity	Total activity	Total minus blank
PP3 + CAP (blank)	0.9	1.33	192	–
PP3 (control)	0.9	3 74	507	315
PP3 + glucose (0.17 M)	2.4	0 34	193	1
PP3 + glucose (0.17 M) + c'AMP 1 mM	2.5	0 43	231	39
PP3 + glucose (0 17 M) + c'AMP 4 mM	2.5	0 42	217	25
PP3 + glucose (0.17 M) + c'AMP 8 mM	2.5	0 44	283	91
PP3 + glucose (0 17 M) + c'AMP 12 mM	2.5	0.44	221	29
PP3 + c'AMP 8 mM	0.9	1 78	263	71

TABLE 3 *Effects of Cyclic AMP on the Synthesis of Hyaluronidase in Non growing Cells*

Medium	pH	Specific activity	Total activity	Total minus blank
PP3	7 4	5 46	755	505
PP3 + c'AMP 1 mM	7 4	4 11	648	398
PP3 + c'AMP 12 mM	7 1	3.95	606	356

cyclic AMP reduced the synthesis of hyaluronidase in non-growing cells in carbohydrate-free medium by almost 50 per cent. However the degree of inhibition varied between experiments and at a concentration of 8 mM cyclic AMP hyaluronidase synthesis in non-growing cells was reduced by 30–50 per cent. Since cyclic AMP decreased the pH of PP3 medium and as synthesis of hyaluronidase is influenced by pH (6) experiments were carried out in order to compare differences in pH and differences in hyaluronidase synthesis in non-growing cultures incubated in PP3 medium and in PP3 medium plus 1 mM or 12 mM cyclic AMP

The results, presented in Table 3 show that differences in hyaluronidase synthesis cannot be explained solely by differences in pH.

The capacity of cyclic AMP at a final concentration of 8 mM to stimulate hyaluronidase synthesis in cells growing in a medium containing 0 06 M glucose was investigated during incubation for 30 min at 37 and 22 C in PP2 medium. The experiments were carried out in order to study the effect of cyclic AMP during the first generation of growth. In order to investigate whether the effect of cyclic AMP was specific in counteracting glucose repression, the effect of an equimolar concentration of 5 AMP on glucose repression was also investigated in these experiments. Three controls were included control for synthesis in PP2 medium at 22 C and controls for repression by glucose in PP2 medium both at 22 and at 37 C.

The results obtained are shown in Table 4 Both cyclic AMP and 5 AMP counteracted glucose repression of enzyme synthesis. Cyclic AMP was more effective in this respect than equimolar 5 AMP

Since cyclic AMP could only relieve slightly the repression exerted by 0 17 M glucose, the difference in specific activity between cultures grown in 0.17 M glucose and in 0 17 M glucose + 8 mM cyclic AMP was studied in 5 independent experiments. The differences, recorded in Table 5 were statistically signif

TABLE 4 *Effect of Cyclic AMP on the Synthesis of Hyaluronidase at Low Glucose Concentration*

Medium	Bacterial concentration relative to the initial (A_t/A_0)	Specific activity	Total activity	Total minus blank
PP2 + CAP 22° C (blank)	0.9	0.64	123	–
PP2 22 C (control)	0.9	3.30	570	447
PP2 + glucose (0.06 M) 22 C	1.3	0.61	159	36
PP2 + glucose (0.06 M) + c'AMP 8 mM 22 C	1 4	1 42	340	217
PP2 + glucose (0.06 M) + 5'AMP 8 mM 22 C	1.3	1.01	217	94
PP2 + glucose (0.06 M) 37 C	1.5	0.29	112	–
PP2 + glucose (0.06 M) + c'AMP 8 mM 37° C	1.5	0.79	251	128
PP2 + glucose (0 06 M) + 5'AMP 8 mM 37 C	1.5	0.63	174	51

TABLE 5. *Difference in Specific Hyaluronidase Activities of Cells Grown on Glucose and on Glucose + Cyclic AMP. Pairs of Observations Made in 5 Independent Experiments*

Specific activity PP3 medium + glucose (0.17 M) + cyclic AMP 8 mM	Specific activity PP3 medium + glucose (0.17 M)	Differences
0.42	0.34	0.08
0.74	0.63	0.11
0.74	0.40	0.34
0.74	0.63	0.11
0.44	0.34	0.10

icant at 5 per cent significance level, tested by Student's t test.

If the results of experiments on the effect of cyclic AMP in relieving glucose repression in media with different glucose concentrations are compared, cyclic AMP seems to be more effective in media of low glucose concentration.

Theophylline, a methyl xanthine derivative, was added to PP3 media containing 0.06 M glucose and cyclic AMP 8 mM. The addition of 1–5 mM theophylline yielded lower hyaluronidase activity as compared to controls without theophylline. Theophylline was also found to inhibit the synthesis of hyaluronidase in non-growing cells incubated in carbohydrate-free medium. This effect was observed even at low concentration (0.5 mM).

DISCUSSION

Enzymes participating in the "peripheral" sequence of the degradative pathway of a carbon source are mainly regulated by control of synthesis in contrast to those of the "central" pathways, the Embden Meyerhof pathway, the tricarboxylic acid cycle, which are regulated by control of activity (2). By the action of hyaluronidase the polysaccharide hyaluronic acid may be brought into the central pathway of glucose breakdown. In fact, N-acetylglucosamine, one of the two subunits of hyaluronic acid, is readily metabolized by this strain of *Streptococcus mutis* if added as the sole carbohydrate to the growth medium (unpublished results).

The regulation of hyaluronidase synthesis by repression described in this study may be termed catabolite repression according to the definitions by *Magasanik* (7) or in some respects transient repression according to the definitions by *Paigen & Williams* (11). However, we will simply refer to the phenomenon as repression of hyaluronidase synthesis and wait until further results are available before another term is suggested.

By comparing the effects of different carbohydrates on the synthesis of hyaluronidase some hypotheses may be formed regarding the identity of the effector or effectors of hyaluronidase repression and the mechanism for the regulation of the enzyme. It is generally considered that, in catabolite and transient repression, the rate of enzyme synthesis reflects the concentration of one or more effectors which may be the growth substrate itself or components of the pool of intermediary metabolites. The concentration of the effector in the cell is dependent (a) on the rate of its entry into the cell or its formation (α) and (b) the rate of its utilization (β). Since the excretion of acid end products of carbohydrate metabolism in this strain was parallel to growth for all carbohydrates tested it seems justifiable to assume that growth rate reflects the rate of utilization of the effector. Growth rate at 37 °C was equal and maximal with glucose and sucrose and it was independent of the exogenous concentration of the carbohydrates. The rate of utilization (β) of the effector controlling hyaluronidase synthesis would then also be equal and maximal (β_{max}) for glucose and sucrose and independent of the exogenous concentration of the carbohydrates. Growth on fructose and mannose was slower and also independent of the concentration of the carbohydrates. The rate of utilization of effector (β_{fru} and β_{man}) would also be less than β_{max}. As mannose which is a glucose analogue completely repressed hyaluronidase synthesis, it seems likely

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POLYSACCHARIDE ACCUMULATION IN *FUSIFORMIS NECROPHORUS*

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Fusiformis necrophorus was studied during growth in stirred fermentors at different pH levels and temperatures. At all studied conditions of cultivation a poly-glucose compound, probably glycogen, was formed by the cells. There was an inverse relationship between cell growth and polysaccharide deposition, maximum glycogen content amounted to 60 per cent of the cell dry weight. In addition, carbohydrate storage was lower at pH 7 than at pH 5.5–6.0 and at 33° C in comparison with 37 and 39° C, indicating that both pH and temperature of the cultivation influence polysaccharide formation. In experiments with accelerated cell growth rate—induced by altered growth conditions—glycogen storage approached zero. It is concluded that *F. necrophorus* possess a previously unrecognized capacity for polysaccharide storage of such magnitude that it should be considered quantitatively in calculations of substrate growth yields.

Fusiformis necrophorus has been studied with respect to its optimal growth conditions (22). In connection with this study the bacteria were found to have the capacity to store glycogen intracellularly. A subsequent study of the energy metabolism of *F. necrophorus* (21) made further information regarding this capacity necessary since it was found desirable to keep the accumulation of intracellular glycogen at the lowest possible level in order to accurately calculate cell yield (Y_G) and cell yield/mole of ATP (Y_{ATP}) of cells grown on glucose. The occurrence of intracellular polysaccharide accumulation is well established for a number of both aerobic and anaerobic bacteria (1, 3, 4, 5, 7, 10, 12, 23) and it is considered to fulfill an important function in energy storage. Quantitatively important amounts, ranging between 20 and 70 per cent of the cell dry weight, have been reported to be accumulated by some species (7, 11, 23).

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No information on intracellular polysaccharide storage in *F. necrophorus* is available, but other bacteria—believed to be closely related—are known to possess this capacity (4). The present study was designed to examine the occurrence of glycogen accumulation in this strain. Primarily, the study was directed towards obtaining maximal amounts of glycogen intracellularly by varying pH and temperature in the culture. This was done in an attempt to delineate some possible regulatory factors for glycogen accumulation in *F. necrophorus*. In the second part of the study it was attempted to suppress the glycogen accumulation by varying the pH, the composition of the medium and the gas mixture introduced into the culture.

MATERIAL AND METHODS

Strain used. *F. necrophorus* (NCTC 7155) Moore and Holdemann (Bergey's Manual of Determinative Bacteriology, 8th edition, in press) was used in this study.

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thin layer chromatography of the hydrolysed material (19) using a silica gel layer and a solvent system containing 65 ml ethylacetate and 35 ml isopropanol H_2O (2:1) Anisaldehyde was used as developing reagent.

Calculations From the determined dry weight (DW) a corrected value (DW) was calculated as the weight of "glycogen free" cells by subtracting from the total cell weight the estimated weight of glycogen. The latter was calculated on the basis of the glucose measurements performed on the polysaccharide hydrolysate. The mean molecular weight of the glycosyl units was taken to be 162 the measured glucose concentration (mg/ml) was thus converted to glycogen concentration by multiplying by the ratio 162/180 The growth rate (GR, mg ml^{-1} h) was estimated from the change in corrected cell dry weight (ΔDW) during a sampling interval (Δt, 1–2 h): GR = $\Delta DW_c/\Delta t$. Similarly the rate of glycogen synthesis (Gly R, mg 100 mg^{-1} h^{-1}) was determined from the change in glycogen content per unit corrected cell dry weight (ΔGly/DW) during a sampling interval (Δt) by the formula Gly R = (ΔGly/DW)/Δt.

RESULTS

In preliminary studies, when *F necrophorus* was grown at 37 C and pH 7 in the basal medium and with gas mixture I significant intracellular accumulation of a polysaccharide could be established. In order to examine the nature of this polysaccharide its composition was determined by thin layer chromatography of the acid-hydrolysed cell polysaccharide. This analysis resulted in the appearance of one single spot on the chromatogram. In addition, the Rf-value for the polysaccharide hydrolysate was identical to that of glucose. It is concluded that the cell polysaccharide is a poly-glucose compound. Although its molecular structure has not been examined it will be referred to as glycogen for discussion see below

Influence of pH on growth and polysaccharide accumulation. Growth and glycogen accumulation of the cells were studied, in the basal medium, at pH 7 0, 6.0 and 5.5 while the temperature was maintained constant at 37 C. Gas mixture I was used. The dry weight of the cells rose markedly and to approximately the same extent irrespective of pH during the first 6–8 h of cultivation

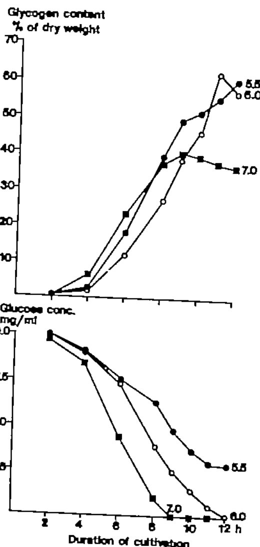


Fig 1 Glycogen content of *Fusiformis necrophorus* grown in the basal medium, expressed as per cent of total cell dry weight (upper panel) and glucose concentration of the culture supernatant (lower panel) during growth at pH 7.0 (■) 6.0 (○) and 5.5 (●) Temperature was kept at 37 C. Mean values for three experiments are given.

(Table 1) This was followed by a small further increment or levelling off of the dry weight for cells growing at pH 6.0 and 5.5 For pH 7 0 a distinct decline in dry weight was seen after 8–9 h of cultivation. The maximum growth rate (0 17–0.21 mg ml^{-1} h) was seen during the first 2–6 h of growth and appeared uninfluenced by the pH.

The glycogen content of the cells reached

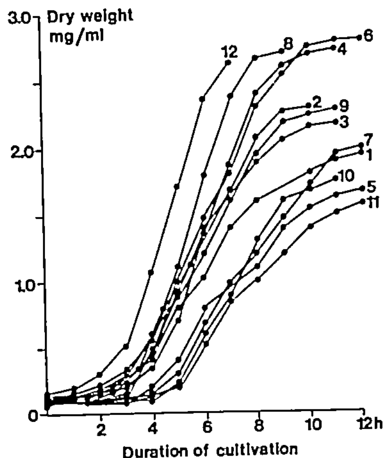


Fig 3 Growth curves of *Fusiformis necrophorus* grown under different conditions. Numbers indicate experiments as described in Table 2.

Studies on minimal glycogen accumulation In an attempt to minimize the glycogen accumulation the cells were grown in the basal medium with the following changes the tryptone concentration was increased to 20 g/L KH_2PO_4 Na_2HPO_4 $7H_2O$ $MgSO_4$ and trace element solution were added and sodium thioglycolate and methylene blue were excluded. Gas mixture II was used and the pH was 6.5 Under these growth conditions, repeated studies showed that the accumulated glycogen was less than 1 per cent of the cell dry weight (Table 2 exp. 12) In subsequent studies these different modifications of the growth conditions were tested one at a time in order to determine which were of significance for the glycogen accumulation.

The findings are presented in Table 2 and Fig 3 The glucose in the cultures was exhausted in some of the experiments (see Table 2) The data given in the table were obtained at the time of maximum glycogen concentration. The results indicate that no single one of the modifications was alone responsible for the reduction in glycogen accumulation observed. The tryptone concentration of the medium appeared to be the most important factor an increase in tryptone concentration from 15 to 20 g/l resulted in an increased growth rate and a ninefold decrease of the glycogen content of the cells (exp. 2) Addition of phosphate-buffer (exp. 3) to the medium and a change of the pH from 5.5 to 6.5 (exp. 8) respectively had similar effects by decreasing the intracellular glycogen approximately fourfold and increasing the growth rate. When gas mixture I was exchanged for gas mixture II (exp. 9) the growth rate was increased and the de-

crease in glycogen content of the cells was approximately 30 per cent. The addition of 3 and 6 per cent oxygen (exp 10 and 11) respectively resulted in a lower growth rate and in a 50 and 65 per cent decrease of the intracellular glycogen concentration. MgSO added to the medium decreased the glycogen accumulation slightly and also decreased the growth rate (exp. 5) This effect of the glycogen accumulation was enhanced when the trace element solution was added at the same time, while the growth rate increased (exp. 6)

DISCUSSION

The present study demonstrates the previously unrecognized capacity of *F necrophorus* to accumulate a polysaccharide during growth. The results from thin layer chromatography of a hydrolysate of the polysaccharide indicate that no monosaccharide other than glucose was present in detectable amounts. Consequently the polysaccharide of the cells is a poly-glucose compound, most likely a glycogen or glycogen-like substances. This hypothesis is further supported by the observed alkaline stable nature of the polymer Alternatively the carbohydrate formed by the cells could be a poly-glucose of dextran type. However this substance is known to appear in the form of delimited capsules surrounding certain of the polysaccharide forming micro-organisms, while others, notably the *Leuconostoc* species, form a diffuse slime covering made up of dextran. Microscopical examination of *F necrophorus* has not revealed the presence of a capsule or a surrounding slime (22) Thus, although the molecular structure of the poly-glucose formed by *F necrophorus* has not been established it appears likely that this substance is glycogen and that it is stored in intracellular granules in agreement with what is known for Escherichia coli (11) Inasmuch as each glycosyl unit of glycogen—except the terminal ones and those at branching points—gain one mole of water during hydrolysis of glycogen, correction for this has been applied in all calculations of glycogen weight based on glucose determinations.

The commercially obtained gas mixture used in this study was not entirely free of oxygen and consequently the culture was not strictly anaerobic. However this should be of little importance in this context since subsequent studies showed that the glycogen accumulation was slightly higher in cultures grown in the presence of trace amounts of oxygen.

In the first part of this study several factors appeared to influence the accumulation of intracellular glycogen. During the initial phase of the cultivation when the growth rate was high, only small amounts of glycogen were formed. In contrast, when the growth rate gradually declined the rate of glycogen formation increased and reached a peak at a time when the growth rate approached zero Thus, an inverse relationship between cell growth and glycogen accumulation was found. This finding is in good agreement with earlier observations that many bacteria can accumulate intracellularly a polyglucose polymer when the growth is limited by nitrogen starvation, low pH, sulphate—or phosphate—deficiency and in the presence of excess carbon source (16) Besides the rate of growth, both pH and temperature of the culture appeared to influence the glycogen formation by the cells. Thus, at pH 7 0 the glucose consumption was rapid but only little glycogen accumulation occurred, the maximum glycogen formation rate was approximately half of that seen for cells growing at pH 5.5 and 6.0 This observation suggests a stimulatory influence of low pH on glycogen formation, particularly since the growth rate at the time of maximum glycogen formation at pH 7 0 was lower than the corresponding values for cells at pH 5.5 and 6.0

The temperature of the culture also seemed to be of importance in this context. The maximum rate of glycogen formation at 37 and 39 C was more than twice that of cells growing at 33 C. Inasmuch as the growth rate was approximately equal and close to zero for all three cultivation temperatures at

the time of maximum glycogen formation the results may provide evidence for an inhibitory influence of low temperature on cell glycogen formation in *F necrophorus*

The question arises how changes in temperature and pH may influence glycogen accumulation of the cells. The biosynthesis of α 1 4-glucosidic linkages of bacterial glycogen occurs by the following reactions

(1) ATP + α-glucose-1 P ⇄ ADP-glucose + PP_i

(2) ADP-glucose + α 1, 4-glucan → ADP + α 1 4-glucosyl-glucan

Reaction 1 is mediated by the enzyme ADP-glucose synthetase and the formation of ADP-glucose appears to be the rate limiting reaction in the glycogen synthesis in bacteria (17) Activators as well as inhibitors of the enzyme ADP-glucose synthetase have been studied in *E. coli* (15) and in species of *Citrobacter Salmonella, Serratia Aerobacter Proteus* and *Erwinia* (17) The effect of temperature on the ADP-glucose synthetase was *not mentioned in those studies. However it is* suggested that the explanation for the adversary effect on glycogen accumulation by low temperature might be an inhibitory effect on ADP-glucose synthetase.

The considerable amounts of glycogen formed by *F necrophorus* under certain growth conditions reaching value as high as 60 per cent of the total cell dry weight are comparable to or higher than values reported for other micro-organisms, i.e. *Arthrobacter* (50–60 per cent, 23) *E. coli* (20–30 per cent, 11 12) and *Ruminococcus albus* (35 per cent, 13) It has been suggested that the intracellular glycogen aids in the preservation of cell integrity during conditions of starvation (16) and can also be utilized as energy source as demonstrated for *E coli* (6) That this is probably the case also for *F necrophorus* is indicated by the findings in preliminary studies using resting cell suspensions of glycogen rich *F necrophorus* in glucose free medium. These cells could be maintained for several hours and a concomitant fall in glycogen content was observed. Cells low in glycogen autolysed rapidly under those conditions. In estimations of bacterial growth yields, storage of glycogen may be of considerable importance (2) Since the energy requirement for glycogen synthesis is smaller (8, 20) than that for synthesis of other nitrogen containing cell constituents the glycogen formed during growth should be taken into account in the calculations of growth yields.

In the second part of the present study it was attempted to minimize glycogen storage. In view of the established inverse relationship between growth rate and glycogen accumulation the growth conditions were modified so as to increase the growth rate of the bacteria. This was achieved by several different alterations. The nitrogen source (tryptone) was added in increased concentration. In addition the medium was supplemented with phosphate ions. $MgSO_4$ and trace elements in combination were found to exert a stimulatory effect on the growth rate and were thus added. An oxygen free environment had a similar effect, thus the oxygen-free gas mixture II was used. The optimal pH for growth of *F necrophorus* was found to be 7.0 (22) but since at this pH the cells had a pronounced tendency to lyse, a slightly lower pH level (6.5) was chosen. Sodium thioglycolate was excluded from the medium since this compound is considered to be slightly toxic (18) In *E. coli* the addition of Mg^{2+} stimulates the ADP-glucose synthetase. The effect of Mg^{2+} is probably *due to its capacity* to complex the ADP which is a product of the reaction and also a strong competitive inhibitor (15) In the present study however addition of $MgSO_4$ (not in combination with trace elements) to the medium decreased the glycogen content as well as the growth rate of the cells slightly The reason for this effect is not clear The yeast extract used in the basal medium contained a low concentration of Mg^{2+} which was probably more than enough to satisfy the needs of the ADP-glucose synthetase. Since the addition of $MgSO_4$ also decreased the growth rate somewhat, the cells were apparently not starved of sulphate when grown in the basal medium. As seen in Table

2 the failure of *F necrophorus* to accumulate glycogen could not be explained by one single modification of the environment but was a joint effect of many factors.

The present study suggests that cell growth rate is the major determinant for intracellular glycogen formation. However direct effect on glycogen synthesis may have been exerted by the modifications of the growth conditions in these experiments, thereby contributing to the reduced polysaccharide formation.

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THE DISTRIBUTION OF ENZYMES AND BACTERIA IN THE SMALL INTESTINES OF SLAUGHTER PIGS

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The activity and distribution of proteinases and their inhibitors, lipases and amylases as well as the composition of the bacterial flora throughout small intestines of 20 slaughter pigs were investigated. It was demonstrated that the intestines were partly in contraction, partly in relaxation and that the contracted regions had no ingesta, only a scarce and mucous content. This type of content possessed no proteolytic activity while lipolytic and starch-splitting activities as well as inhibitory substances against proteinase were always present. The contracted parts, which presented no regularity as to their length and localization, often appeared to be sterile. In the feed-containing, relaxed regions of the intestines, the digestive enzymes examined were always present in high concentrations. Their activity was quite constant in all segments of the intestine. The proteolytic activity was partly based on trypsin. The main microorganisms found were *Lactobacillus* spp., α-haemolytic streptococci and lactosepositive enterobacteria (coliform). The quantitative bacteriological findings varied considerably.

Some work has been performed with regard to the distribution and the activity of digestive enzymes in the alimentary tract of man, some laboratory animals and dog (*Borgström et al.* 1957, *Kickens* 1963). As far as farm animals are concerned little information seems to exist on this subject.

The composition of the normal bacterial flora in the intestines of man, laboratory animals and also some domestic animals has been investigated by numerous authors (*Dixon* 1960, *Smith & Jones* 1963, *Månsson* 1966, *Ishikawa* 1972, *Kovács et al.* 1972, *Mallory et al.* 1973). Comparison of the microbial content qualitatively as well as quantitatively has, to some extent, been performed for various animal species including man (*Bornside & Cohn* 1965). The microbial flora in the colon and faeces has been most thoroughly investigated. The relationship between the microbial content, enzyme activity and the intestinal content in the various parts of the small intestines does not seem to have been investigated to any large extent.

In order to understand the pathogenesis of certain intestinal infections it was considered necessary to obtain more information about the normal conditions in the intestines. Thus, the aim of the present work was to investigate the distribution of some digestive enzymes in the small intestine of slaughter pigs, and to compare the enzyme distribution with the intestinal content as well as with the bacterial flora in the various parts of the small intestines. It was also found to be of importance

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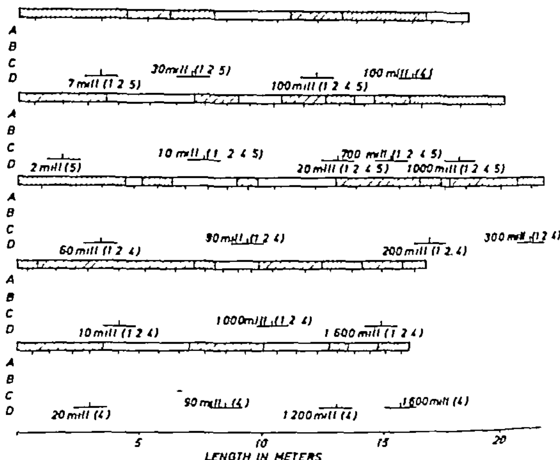


Fig 1 Distribution of different types of intestinal content, proteolytic enzymes, proteinase inhibitors and coliform bacteria, as well as the total number of bacteria in five different small intestines.

the presence of alternating contracted and relaxed parts. The number localization and length of the contracted and relaxed regions varied from one intestine to another In Fig 1 the distribution of the various regions in five different intestines is indicated schematically In the contracted regions there were practically no ingesta the content was scarce and of mucous or "mayonnaise like" consistency The relaxed regions contained a varying amount of digested fodder and the content differed from a watery or "soup-like" to a mucous and "porridge like" or pasty consistency The border between the feed-containing, relaxed regions and the contracted regions could be very distinct and sharp (Fig 2)

The pH of the intestinal content ranged

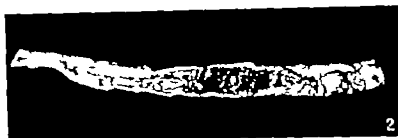


Fig 2 Part of a small intestine with a distinct border between feed-containing and mucous-containing regions.

DUODENUM

ILEUM

Log 10 DIFFUSION UNITS/0.050 ml OF UNDILUTED INTESTINAL CONTENT

A PROTEOLYTIC ENZYMES

B ELASTASE

C INHIBITORS AGAINST PROTEOLYTIC ENZYMES

D LIPASE

E AMYLASE

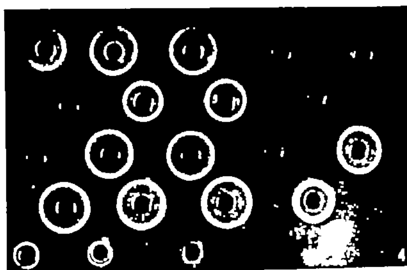
CONTRACTED REGIONS WITH SCARCE MUCOUS AND "MAYONNAISE LIKE" CONTENT

RELAXED REGIONS WITH A WATERY OR SOUP-LIKE CONTENT

RELAXED REGIONS WITH A PASTY AND PORRIDGE LIKE CONTENT

PLACES FROM WHICH SAMPLES WERE TAKEN

Fig 3 Distribution of enzymes, and proteinase inhibitors in one small intestine (Second from the top in Fig 1)



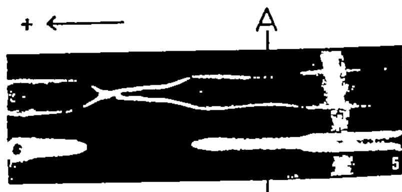


Fig 4 Casein precipitating activity of samples—from upper left—from the small intestine (1 17) from the large Intestine (18–20) and from the ventricle (21) (The second Intestine from the top in Fig. 1)

Fig 5 Electrophoretic CPI-test of serum from rabbit immunized with swine trypsin. Intestinal contents used are (downwards) undiluted and diluted 1 100. The inhibiting effects of the specific antibodies to swine trypsin appear near the line of application (A) while the naturally occurring inhibitors are located towards the anode. The electrophoresis was carried out in 0 05 M phosphate buffer at pH 6 2 for 18 h at 1 0 V

between 4 and 6 in the duodenum and anterior portion of the jejunum, while in other parts of the small bowel the values were relatively constant around pH 6.5 occasionally increasing to pH 7.5 in the lower areas.

Bacteriological investigation. The bacteria found could be classified into following groups

1 *Lactobacillus* sps.
2 a haemolytic streptococci.
3 *Micrococcus* sps.
4 Lactosepositive enterobacteria (coliforms)
5 *Bacillus* sps. (aerobic sporeforming bacteria)
6. *Clostridium perfringens*
7 *Bacteroides* sps.

Organisms of one of more of the mentioned groups were also found in the ventricular content, and those dominating in the ventricular content were also the main organisms in the anterior part of the small in-

testines. The bacteriological findings in the various parts of five intestines in relation to the intestinal content and the presence of proteolytic enzymes and proteinase inhibitors are presented in Fig 1

In the regions of the intestines with no ingesta but only a scarce "mayonnaise-like" mucous content, coliform bacteria never occurred, even though such bacteria were found in large numbers in the feed-containing regions on both sides (Fig 1) If bacteria were found in the mentioned regions they belonged to groups 1 and 2 and in such cases they were present in a very small number In the feed-containing regions the coliform bacteria were the most common, especially in the posterior part, and in many intestines these organisms dominated throughout the whole length. The number of coliforms increased towards the posterior end of the small intestine. The other groups commonly found in relatively large numbers were lactobacilli and α-haemolytic streptococci which in some cases were the only organisms found in duodenum. Aerobic sporeformers and micrococci were found in considerable numbers in some cases, but mostly only in undiluted intestinal content.

Clostridium perfringens occurred in small numbers in the posterior parts of the small intestines in about 60 per cent of the intestines examined. In none of the cases was the organism present in sufficient number to be demonstrated in the diluted samples. Asporogenic Gram-negative strictly anaerobic rods (Bacteroides) were sporadically isolated—in one case also from the anterior part of the jejunum—in relatively low numbers (150000-200000 organisms per gram content)

The quantitative bacteriological investigations revealed some variation between the various intestines as can be seen in Fig. 1

Enzymological investigation. The qualitative and quantitative distribution of the various enzymes in relation to the intestinal content in one representative intestinal tract is presented in Fig. 3 Fig. 1 illustrates the qualitative distribution of proteolytic enzymes in five different intestines in relation to the intestinal content and the presence of coliform bacteria. It is evident that in parts with no feed content, but only a "mayonnaise like" cover no proteolytic activity could be demonstrated. The presence of elastase parallels

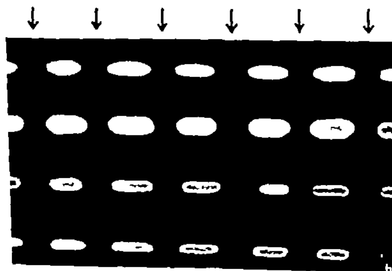


Fig 6. Crossed-un CPI-test with mucous and "mayonnaise-like" content (marked by arrows) from the samples of the small intestine showing no proteolytic activity in Fig 3 and Fig. 4 The enzymes used are (downwards) Swine trypsin (0 01 mg per ml) α-chymotrypsin (0.01 mg per ml) bovine trypsin (0 01 mg per ml) and proteinases from *Bacillus subtilis* 0.005 mg per ml)

TABLE 1 *Quantitative Determination of Proteolytic Activity by the Kunitz Method Compared with the Semi-quantitative CP-test in One Representative Small Intestine*

Sample number	O D at 280 nm (Kunitz method)	Zone diameters in mm by the CP test
1	0.585	13
2	1.215	18
8	0 703	18
12	1.590	20
13	1.200	20
16	2 400	21
17	1.585	20
18	0.225	12

the presence of proteinases. The proteinase activity was of approximately the same size in the various feed-containing parts, both with regard to the samples taken in the anterior the middle or the posterior part of the jejunum. The proteinase activity in colon was negligible. The CP-activity in various samples taken from one representative intestinal tract is shown in Fig 4 The electrophoretic CPI test with antiserum against swine trypsin regularly resulted in an incomplete inhibition of the CP-activity in the various samples (Fig 5) Quantitative determination of the proteolytic activity performed by the Kunitz method gave results similar to those obtained by the CP test (Table 1) In areas of the intestines containing no feed, no proteinase or elastase activity and no coliforms, inhibitors against proteolytic enzymes were found. The effect of the inhibitory material upon various enzymes is shown in Fig 6 In addition to inhibiting the intestinal proteinases, the inhibitors were also active against bovine and swine trypsins, bovine chymotrypsin and various microbial proteinases. The inhibitory activity could be demonstrated in dilutions up to 1 80 of the "mayonnaise-like" content.

Lipase and amylase activities were, in contrary to the proteolytic activity present in all parts of the small intestines. The activity of amylase was, however far less in the empty parts of the intestines than in the relaxed parts containing digested fodder

Histological investigation A comparison of the histological studies of the intestinal mucosa in the contracted and the relaxed areas revealed no qualitative differences.

DISCUSSION

The present investigation revealed the existence of a varying number of alternating contracted and relaxed parts of the small intestines in slaughter pigs. The border between the relaxed and contracted regions were usually sharp. Their content differed qualitatively as the contracted parts only contained a mucous cover which often appeared to be sterile. Neither coliform bacteria nor proteolytic activity could be demonstrated in this content. These findings may indicate that the normal peristaltic movements of the intestines cause a "washing out" of the intestinal content. The production by the epithelium of the mucous cover is probably also of importance for the removal of bacteria and some enzymes. The resting period, although it is short, obtained in this way may be beneficial or even necessary for the regeneration or recovery of the epithelium before the next portion of intestinal content enters the region. In some disease conditions, for instance infections caused by enterotoxigenic *Escherichia coli*, this organism is found in large amounts throughout the small intestine. In this case the types of *Escherichia coli* involved seem to adhere to the epithelial cells because of their K-antigens (*Smith & Halls* 1968, *Arbuckle* 1970) Thus they avoid being "washed out" even though the peristaltic activity is unaffected. The non-enteropathogenic types of *Escherichia coli* on the contrary are present in the lumen or luminal content (*Smith & Halls* 1968, *Arbuckle* 1970) and are thus transported together with the content.

It is, however a striking phenomenon that the elimination of proteinases and bacteria is so complete, and that the border between bacteria and proteinase-containing and bac

tern- and proteinase-free regions is that sharp. It is possible that not only the "washing out" effect due to the peristaltic movement—with considerable reduction of proteinases—but also a general production of proteinase-inhibitors in the intestinal mucosa explains the absence of proteolytic activity in these contracted regions. Thus—in contrast to the feed-containing parts, the inhibitors may be present in excess and lead to an inactivation of proteinases possibly still present. The presence of proteinase inhibitors in the intestinal content of man and dog has previously been demonstrated by *Hochstrasser et al.* (1972) however without reference to the distribution of enzymatic activity and the character of the content.

Although proteolytic activity is completely absent in the contracted parts, amylase and lipase activities exist to a considerable extent. Considering the effectiveness of the peristaltis in draining the intestine, it is not certain that these enzymes originate from pancreas. The possibility that amylases and lipases in the regions mentioned originate from the intestinal wall itself either from the epithelial cells, the tissue fluid or from the blood, cannot, therefore, be excluded. Animal sera reveal both starch-splitting and lipase activities. This observation together with the demonstration that the inhibitory spectrum and the electrophoretic patterns of the intestinal proteinase inhibitors seems to be the same as for those in serum (*Fossum* 1970 b) further support the theory that amylase and lipase activities in the contracted parts do not necessarily originate from pancreas.

One explanation for the fact that coliform bacteria are not present in the above mentioned regions could be the presence of a bactericidal effect in the mucous cover. Bactericidal activity was, however not demonstrated. This is in agreement with the results obtained by other workers. (*Goldsworthy & Florey* 1930 *Sullivan & Mascilla* 1937 *Dixon* 1960)

In the relaxed feed-containing regions of the small intestines, bacteria and digestive enzymes, including proteinase and elastase were always found. The partial inhibition obtained by the electrophoretic CPI test with antiserum against swine trypsin indicates that trypsin to a considerable extent may be responsible for the proteolytic activity throughout the whole small intestine. The activity of the digestive enzymes investigated was approximately of the same order per unit volume of intestinal content in the various relaxed regions throughout the jejunum, although the activity varied for different intestines. This finding does not exclude a continuous inactivation of enzymes—for instance due to the presence of inhibitors—during the passage of the content through the intestine as the content becomes more concentrated in the posterior parts.

The observation that the number of bacteria increased towards the posterior end of the jejunum is in agreement with observations made previously (*Dixon* 1960 *Smith & Jones* 1963 *Månsson* 1966, *Kovács et al.* 1972) In humans there seem to be a small number of bacteria in the ventricle and duodenum, (*Bornside & Cohn* 1965) In the present investigation, large amounts of bacteria were usually found in these areas, a fact that may be explained by the higher pH in the ventricular content of pig compared to that in man.

When considering the present findings some reservations should be made. First, one should be aware of the possibility of some alterations of the in vivo intestinal conditions during the slaughtering process. Secondly it should be remembered that the influence of feeding methods (number of feeding each day dry or wet feeding, water availability) and of feeding material as well as time between last feeding and slaughtering on the bacterial and enzymatic conditions are not included in the present work. It should also be emphasized that the sensitivity of the various methods used for determination of enzyme activity varies greatly

The present investigation shows that the biologically active components in the alimentary tract form a very complicated system. More knowledge about the functions and

relationship of all these factors will certainly be of importance for the understanding and thus for prevention of certain pathological conditions in the intestinal tract.

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swabs which were inserted into the pelvis through an incision. In addition after cauterization of the kidney surface a column of tissue including the renal cortex, medulla and papilla was cut out, using a needle with a diameter of 5 mm. The three zones of tissue were homogenized separately in a Potter-S homogenizer.

Cultivation for Mycoplasmas and Bacteria

Large-colony mycoplasmas. The medium used was the B medium (6). Swabs, urine and homogenate of kidney tissue were inoculated in semisolid B-medium and streaked onto duplicate sets of B-plates. *One set was incubated at 37 C in* candle-jars. The other set was incubated in an atmosphere of 95 per cent N_2 plus 5 per cent CO_2. Cultures in the semisolid B-medium were incubated at 37 C and subcultivations on B-plates were performed after three days. The plates were read under a stereomicroscope after 4 and 8 days of incubation. For further details of the cultivation procedure reference is made to a previous paper (16).

U. urealyticum. For cultivation of *U. urealyticum* the specimens were inoculated in broth and on duplicate sets of S-plates (2). The plates were incubated at 37 C in 90 per cent atmospheric air plus 10 per cent CO and in an atmosphere of 95 per cent N plus 5 per cent CO_2, respectively. The broth was incubated at 37 C for 24 hours after which subcultivation on plates was performed. Examination for growth was performed under a stereomicroscope after 3 and 5 days of incubation.

Bacteria. All specimens were cultivated for bacteria on blood agar, lactose bromthymole blue- and chocolate agar plates.

Identification of Isolates

The isolated mycoplasma strains were cloned and thereafter grouped according to their ability to catabolise glucose and arginine, reduce tetrazolium, produce phosphatase, and hydrolyse urea.

Final identification was performed by the disc growth inhibition (DGI) (2, 4), indirect immunofluorescence (IMF) (1, 13) and metabolic inhibition (MI) (1, 12) tests using antisera from the FAO/WHO International Reference Centre for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus.

RESULTS

Mycoplasmas Recovered from the Urethra

Mycoplasmas were isolated from the urethra in 9 cases (4♂ and 5♀) (Table 1).

Eight of the isolates were cultivated under both of the atmospheric conditions used. Three of these isolates were identified as *M. hominis*, one as *M. fermentans* and 4 as *U. urealyticum*.

The remaining strain (A53D) was only isolated on a plate incubated at 95 per cent N_2 plus 5 per cent CO_2 following subcultivation from the primary culture in semisolid medium. The strain hydrolysed arginine and was phosphatase positive, but did not ferment glucose or reduce tetrazolium aerobically or anaerobically. This biochemical pattern is identical with that of *M. primatum* (5) and the isolate was subsequently serologically identified as this species by DGI, IMF and MI tests, using antiserum against the type strain (HCR292) (Table 2). No crossreactions with antisera against other human mycoplasmas or animal strains with a similar biochemical pattern were observed.

Mycoplasmas Recovered from the Bladder

U. urealyticum was cultivated from the bladder urine in 2 out of 15 cases (Table 1) in one of these cases together with *Streptococcus faecalis*. None of the patients harbour

TABLE 1 *The Post Mortem Mycoplasmal Flora of Urethra, Bladder and Upper Urinary Tract of 19 Men and 21 Women*

Localization	Number of cases investigated	No. of cases positive for								Total No. of positive cases
		M. hominis		*M. fermentans*		*M. primatum*		*U. urealyticum*		
		Men	Women	Men	Women	Men	Women	Men	Women	
Urethra	40	1	2	0	1	0	1	3	1	9
Bladder	15	0	0	0	0	0	0	2	0	2
Upper urinary tract	40	0	0	0	0	0	0	1	0	1

TABLE 2. *Results of Serological Tests Using as Antigen Strain A53D and the Type Strain of M. primatum and Antisera Against the Type Strain of M. primatum (HRC292)*

Mycoplasma species	Titres of serological tests		
	Disc-growth inhibition (mm)	Metabolic inhibition	Indirect immunofluorescence
Strain A53D	4	512	+ + +
M. primatum (HRC292)	5	512	+ + +

ing mycoplasmas in the bladder had revealed clinical signs of urinary tract infection.

Mycoplasmas Recovered from the Upper Urinary Tract

The only isolate obtained from the upper urinary tract was identified as *U. urealyticum* type I. This strain was cultivated from the cortical zone of the left kidney of a 79-year old man. There was no clinical history of urinary tract infection, and no history of antibiotic treatment, but histological examination of the kidney showed diffuse subcapsular infiltration with mononuclear leucocytes. Furthermore, a chronic non-specific inflammation was found in the papilla. The same serotype of *U. urealyticum* was isolated from the bladder urine (10^3 c.f.u./ml) and the urethra. Bacteria could not be cultivated from the kidneys or from the bladder urine of this body.

DISCUSSION

The results of cultivation for mycoplasmas from the urethra and bladder in this autopsy study are, with one exception (the isolation of *M. primatum*), in general agreement with those obtained from patients of similar age groups (7, 13, 17).

The isolation of *M. primatum* is noteworthy in as much as this species has been demonstrated only once before in man, viz. from an inflammatory skin lesion of the umbilicus (14). On the other hand, *M. primatum* is a frequent parasitic inhabitant of the urogenital tract and oral cavity of monkeys (5).

It may be mentioned that other strains of *M. primatum* were not handled in this laboratory when the present study was carried out.

Inflammatory lesions were only found in the kidneys of 5 of the examined bodies, and the only mycoplasma isolate from the upper urinary tract was cultivated from one of these kidneys. Thus, the results of cultivation for mycoplasmas from the upper urinary tract were sparse, but they are on a par with those obtained in other studies concerning a probable connection between mycoplasmas and pyelonephritis (11, 16, 17).

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MUTATIONS IN ARTHROBACTER AFFECTING THE FORMATION OF EXTRACELLULAR PROTEASE

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Seventeen mutants of a strain of *Arthrobacter* producing altered amounts of extracellular protease were isolated. One mutant had lost the ability to form wild type extracellular protease. Despite this loss, the mutant demonstrated proteolytic activity due to leakage of a thermolabile intracellular proteinase. Chemical, immunological and catalytic properties of this enzyme show that it is not related to the extracellular protease of the wild type.

Many kinds of bacteria secrete extracellular proteases, and some of these enzymes have been studied with respect to chemical and catalytic properties. There are numerous reports on the effect of varied culture conditions on bacterial protease production, but the exact mechanisms which regulate the synthesis and secretion of these enzymes are still largely unknown. High concentrations of carbohydrates or amino acids usually repress enzyme formation, indicating catabolic repression as a regulatory mechanism. (*Chan & Chaloupka* 1972, *Schaeffer* 1969, *Tanaka & Iuchi* 1971). On the other hand the presence of proteins or peptide mixtures often enhance the protease synthesis, and this has been interpreted as evidence of induction (*Dahle* 1971). The cell membrane and mesosomes have been suggested as sites of synthesis of extracellular enzymes, and the secretion process is probably associated with membrane growth (*Lampen* 1965).

A strain of *Arthrobacter* isolated in our laboratory has been shown to produce a truly extracellular protease (*Hofsten & Tjeder* 1965). The enzyme is secreted during exponential growth, and there is no enzyme with similar properties in cell extracts. However, the cell extracts contains a different kind of protease and several exopeptidases (*Bjare et al.* 1970, *Rydén* 1971).

To study the formation of extracellular protease in *Arthrobacter* we isolated seventeen mutants which differed in their ability to secrete extracellular protease. Two of these mutants produced extracellular protease which was heat labile. This paper reports on the characterization of one of these mutants and describes physical and chemical properties of the protease produced.

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MATERIALS AND METHODS

Bacterial strains. The wild type *Arthrobacter* strain B22 was originally isolated from a grass infusion (*Hofsten & Tjeder* 1965). Most of its culture characteristics are similar to those described in Bergey's Manual for *Arthrobacter ureafaciens*. The mutant strain B22.1 described in this paper was isolated from a UV-irradiated culture of strain B22. Both strains were maintained on slants of Difco Nutrient agar and stored lyophilized.

Media and growth conditions. Extracellular proteolytic activity was detected by plating on milk agar made by mixing equal volumes of double concentrated Nutrient agar and an autoclaved solution of 2 per cent (w/v) non-fat milk powder in water. This medium is turbid and colonies of proteolytic bacteria are surrounded by clear halos due to hydrolysis of the casein. The plates were examined after two days at 30 C, and colonies with halo diameters different from the average were isolated and further tested by growth in liquid media. The liquid growth medium had the following composition K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.1 g, $CaCl_2$ 10 mg, $FeSO_4 \cdot 7H_2O$ 0.5 mg, Difco Gelatin 10 g and Difco cantone 1 g in 1000 ml distilled water. pH was adjusted to 7.5 before autoclaving. Bacteria were grown either in Erlenmeyer flasks at 30 C on a shaking incubator or in 25 litre glass flasks fitted with stirrers to provide adequate suspension of the cells. The 25 litre culture was aerated with 150 litres of air per hour and 0.5 ml Antifoam A was added per 15 litre culture.

Mutagenization and isolation of mutants. Cells grown in Difco Nutrient Broth over night at 30 C were harvested by centrifugation, washed once in sterile buffer pH 7.4 and diluted to approximately 10^9 cells/ml in the same buffer. Ten millilitres of the cell suspension in a 9 cm petri dish was irradiated for 60 seconds at a distance of 35 cm with a Philips (TUW 15 W) mercury vapour tube, emitting about 90 per cent of its light at 2537 Å. This treatment resulted in 1–5 per cent surviving cells. The irradiated cell suspension was added to an equal volume of double concentrated Nutrient Broth and incubated for 20 hours to permit expression of the culture phenotype. After dilution, the cells were plated on milk agar t give about 50 colonies per plate.

Determination of enzyme activities. Protease activity of cell-free media was measured using one percent casein (Merck) dissolved in 0.1 M Tris-HCl buffer pH 8.0 as substrate. One ml of substrate and one ml of enzyme solution were incubated at 37 C for one hour. The hydrolysis was stopped by adding 3.0 ml of 5 per cent trichloroacetic acid (Merck) the precipitate filtered off and the absorbance of the filtrate measured at 280 nm. One unit of protease is arbitrarily defined as the amount of enzyme which gives an increase in absorbance of 0.1 per hour.

A direct spectrophotometric assay for determination of protease activity was also used (*Beyer & Carlton* 1968). Hydrolysis of peptide bonds in proteins was recorded as a decrease in absorbance at 235 nm measured in a Hitachi 1 4 double beam photometer with a thermostated cuvette holder. A solution of 0.002 per cent casein (Merck) in 0.02 M potassium phosphate buffer pH 7.4 was used as substrate and measurements were performed at 37 C.

Hydrolysis of ester bonds was followed at 37 C by measuring the increase in absorbance at 256 nm with the same photometer (*Beyer & Carlton* 1968). The esters acetyl-L-tyrosineethylester, ATEE, and N-benzoylarginine ethylester BAEE, (Nutritional Biochemical Corp. USA) were used as substrates at a concentration of 1 mM in 0.1 M Tris-HCl buffer pH 8.0.

Hydrolyses of dipeptides was determined with the ninhydrin method of *Matheson & Tattrie* (1964) at a substrate concentration of 0.1 per cent in 0.1 M Tris-HCl buffer pH 8.0, with 5 mM $MgCl_2$. The following peptides were tested: leu-gly, gly-leu, gly-gly and ala-gly. Hydrolysis of poly-L-lysine was determined by thin-layer chromatography on silicagel G in n-butanol:pyridine:acetic acid:water (30:24:6:30) after incubation at pH 8.0 for two hours at 30 C.

Enzyme preparation. For preparation of enzyme from culture liquids of strain B22 1 the cells were harvested by centrifugation after 15 to 18 hours of growth.

Extracellular enzyme from cultures of B22-1 was concentrated by adding 10 g of cellulose powder (Whatman) per litre of culture filtrate and $(NH_4)_2SO_4$ to 70 per cent saturation. After 15 hours, the suspension was centrifuged and precipitated protein eluted from the cellulose by 0.1 M Tris-HCl buffer pH 8.0 containing 5 mM $MgCl_2$. Protein was reprecipitated by adding $(NH_4)_2SO_4$ to 45 per cent saturation. After 15 hours, the precipitate was dissolved in 0.1 M Tris-HCl buffer, pH 8.0, containing 5 mM $MgCl_2$ and dialysed against the same buffer for 20 hours.

Cell-bound protease was prepared from cells grown until the late exponential growth phase. The cells were washed twice and suspended in 0.1 M Tris-HCl buffer pH 8.0, with 5 mM $MgCl_2$. Cell density was adjusted to about 5×10^{10} bacteria per ml. Twenty ml samples of this suspension were treated in a Raytheon 10 Kc sonic oscillator for 20 min at 7 C and centrifuged at 33,000 × g for 60 min to remove remaining cells and cell debris.

Fractionation methods. Column chromatography was performed using Sephadex G-100 and DEAE-Sephadex A 50 (Pharmacia Fine Chemicals, Uppsala). The buffer used was 0.1 M tris-HCl, pH 8.0, with 5 mM $MgCl_2$. Proteinase was eluted from the DEAE-Sephadex either by stepwise or gradient elution with NaCl.

Column zone electrophoresis was performed according to *Porath* (1956) on a vertical column packed with Sephadex G-25 in Tris-citrate buffer pH 8.6, at an ionic strength of 0.075.

Isolation of phages. Two different phages, lytic against *Arthrobacter B22* were isolated by mixing samples of garden soil with a culture of bacteria growing in Nutrient Broth. After incubation for 15 hours at 30° C, the culture was centrifuged at

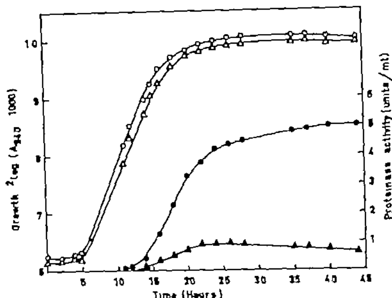


Fig 1 Growth (open symbols) and protease production (closed symbols) in cultures of B22 and B22 1 grown in gelatine medium at 30 C. Growth is illustrated as increase in ^{2}log of absorbance (A_{540nm} × 10^3)
○: B22, △ B22 1

15,000 × g for 15 min and the supernatant passed through a 0.45 μm Millipore filter. Phage titres were determined with standard phage methods using a bottom agar of Difco Nutrient Agar. The top agar had the same composition as the bottom agar except for the agar concentration which was 0.7 per cent. Phage stocks were prepared by the soft agar overlay method and freed from bacteria by filtering through Millipore filters.

Immunological methods. The following antisera were used *Antiserum A* prepared by injecting heat-killed or formaldehyde-treated cells of *Arthrobacter B22* into rabbits. *Antiserum B* was prepared by injecting pure, crystalline extracellular protease from the wild-type into rabbits. *Antiserum C* was prepared with a cell extract of the wild-type as antigen. Antisera A and B were prepared by Dr *S* Dafis, Astra Pharmaceutical Co Södertälje and antiserum C was prepared by Dr *U Bjare* at the Institute of Biochemistry Uppsala.

Antiserum A was used in agglutination tests. Two drops of serum diluted 1 10 in 0.9 per cent NaCl were mixed with two drops of a suspension of washed bacteria on glass slides. The cell density of the tested suspensions was about 10^{10} cells per ml.

Antisera B and C were used in gel diffusion and immunoelectrophoresis experiments. Gel diffusion was made with 0.8 per cent Difco Noble Agar dissolved in 0.9 per cent NaCl. Immunoelectrophoresis was performed on microscope slides with 1 per cent Agarose (Behringwerk AG) dissolved in Veronal buffer pH 8.6, with an ionic strength of 0.1 The electrophoresis was run for 45 min at 50 V Concentrated culture media, used as antigens, were made by growing bacteria for 18 hours and lyophilizing the cell-free culture liquid. The dried material was dissolved in water to a concentration ten times greater than the original culture liquid.

RESULTS

Isolation of mutants. The methods used for screening of mutants allowed isolation of 17 mutant strains of *Arthrobacter* with changed ability to produce protease. No attempts were made to determine the frequency at which mutants occurred, but several thousands of colonies were examined for each mutant found.

One of the mutant strains, called B22 1 was selected for further study because the halos round colonies on milk agar were not only smaller than the halos round colonies of the wild type, but were also turbid, indicating incomplete hydrolysis of casein.

Spontaneous wild type revertants of strain B22 1 were never discovered. Mutagen treat

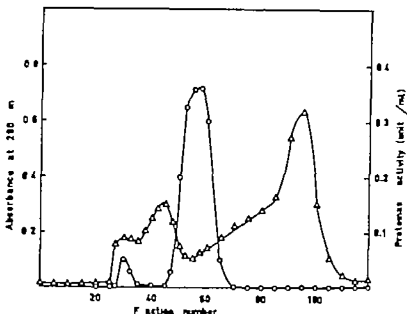


Fig 2 Gel filtration on Sephadex G-100 of concentrated culture filtrate of B22 1 grown in Medium 59 Buffer 0.1 M Tris-HCl pH 8 0 5 mM $MgCl_2$. Column dimension 3 cm × 90 cm.
△: Material distribution. ○: Protease activity

ment (UV irradiation and ethyl-methane-sulphonate) was also tried to induce reversion but with negative results.

All strains were tested against antiserum A and found to be agglutinated in the same manner as the wild type whereas various other Gram-positive bacteria including other species of *Arthrobacter* gave negative reactions. The phages isolated against the wild type were active against all mutants.

Growth and enzyme formation in strain B22 1 Fig 1 shows that there was no difference in growth rates of the strain B22 and B22 1 in the gelatin medium, but that cultures of the mutant had only 10–20 per cent of the proteolytic activity of wild type cultures. After 24 hours, when both strains had reached the stationary growth phase, there was no further increase in protease activity in the mutant culture.

The protease activity of the mutant was reduced about fifty per cent, if the culture or the cell free growth medium was incubated at 55 C for 60 minutes, whereas similar treatment of a culture of strain B22 did not affect its protease activity

The two strains were grown in the gelatine medium and samples were removed at different times during the growth cycle. The cells were washed twice and suspended in 0.1 M *Tris-HCl buffer pH 8 0* with 5 mM $MgCl_2$ and immediately assayed for protease activity No activity was found in any of the suspensions.

Cell suspensions of the two strains in 0.1 M TrisHCl, pH 8.0 (2 mg/ml dry weight) were also tested for leakage of UV-absorbing material when incubated in 0.1 M Tris-HCl, pH 8.0 buffer for one hour at 20 C. The mutant cells were found to release about three times as much UV absorbing material compared with the wild type cells. This shows that B22 1 has abnormal permeability properties. No protease activity was released by any of the strains.

Preliminary purification experiments. In order to compare the biochemical properties of the extracellular protease with those of the wild type, various fractionation experiments were made. The normal wild type enzyme was isolated as described earlier (*Hofsten et al.* 1965) but it was not possible to purify

TABLE 1 *Characteristics of Prot ases from Arthrobacter B22 and B22 1*

	B22 extracellular protease	B22 1 extracellular protease	B22 1 intracellular protease
Elution volume, Sephadex G-100 (Fig. 2 and 3)	2.5 × V	1.8 × V	1 7 × V
Charge, pH 8.6	positive	negative	negative
DEAE-Sephadex chromatography pH 8.0	not adsorbed	adsorbed	adsorbed
Inhibition by EDTA	no	yes	yes
Stability 55 C, pH 8.0	yes	no	no
Activity against poly-L-lysine	yes	no	no
Reaction with antiserum against B22 protease	yes	no	no
Reaction with antiserum against cell extracts of B22	no	yes	yes

the enzyme from strain B22 1 by the same procedure because of inactivation of this enzyme. When 15-litre cultures of the mutant were grown for 18 hours at 30 C, the protease activity was 0.25–0.50 units per ml at a cell density corresponding to about 0 7 mg dry cells per ml. Ultrafiltration and freeze drying were tested as methods for concentration of the protease activity of centrifuged cultures, but little activity was recovered. Precipitation with ammonium sulphate as described in *Material and Methods* was therefore used and the solution obtained was then used for further fractionation experiments. The results of these are described below and summarized in Table 1 which also contains comparative data on cell bound protease of B22 1 and the extracellular protease of the wild type.

Gel filtration experiments. Fig 2 illustrates a gel filtration experiment with the extracellular protease of B22 1 concentrated by ammonium sulphate precipitation. The protease activity was separated into two peaks, called P1 and P2 respectively P1 appeared with the high molecular weight material eluted with the void volume and P2 appeared 0.8 void volume later No protease activity was detected 1.5 void volumes after the unretarded peak, where the extracellular protease of the wild type appears when present (*Hofsten et al.* 1965)

Gel filtration of cell extracts of B22 1 under the same experimental conditions separated the proteolytically active material in a similar way as illustrated in Fig 3 One peak was eluted with the void volume, and a second peak appeared 0.7 void volume later Dipeptidases present in cell extracts of *Arthrobacter* (*Bjare et al.* 1970 *Rydén* 1971) are only slightly retarded from and follow close behind the void volume. These enzymes cannot therefore cause the proteolytic activity eluted 0.5 void volumes later

Ion-exchange chromatography The wild type extracellular protease is not adsorbed on DEAE-Sephadex A 50 at pH 8.0. (*Hofsten et al.* 1965) In contrast, we found that the extracellular mutant protease was adsorbed. The ion-exchanger was equilibrated with 0 05 M Tris-HCl buffer pH 8.0 with 5 mM $MgCl_2$ and dialysed enzyme was applied to the column. No proteolytically active material was eluted with the starting buffer Protease could however be eluted by either step-wise (Fig 4) or gradient elution with NaCl. The recovery in these experiments was between 10 and 20 per cent.

When dialysed cell extracts of B22 1 were applied to the DEAE-Sephadex under the same conditions, the protease activity was also adsorbed. In this case protease activity was also eluted with 0 4 M NaCl, but the recovery never exceeded a few per cent.

Zone electrophoresis. Fig 5 illustrates column zone electrophoresis of both extracellular

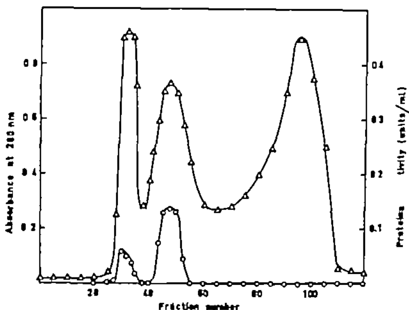


Fig 3 Gel filtration on Sephadex G-100 of cell extract of B22 1 Conditions and symbols as in Fig. 2.

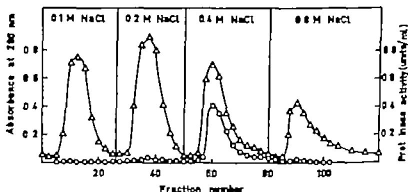


Fig 4 Chromatography on DEAE-Sephadex A 50 of extracellular protease of B22 1 The material was applied in 0.05 M Tris-HCl buffer pH 8.0 5 mM $MgCl_2$. Elution was performed stepwise.
Δ Material distribution. ○ Protease activity

and intracellular protease of B22 1 at pH 8.6. Both enzymes moved towards the anode and were eluted as single peaks. The extracellular and the intracellular proteases of the mutant were negatively charged at pH 8.6 whereas the wild type extracellular protease is positively charged at pH 8.6 (*Hofsten et al* 1965)

Catalytic properties Hydrolysis of peptide bonds results in a decrease in absorbance at 235 nm (*Boyer & Carlton* 1968) This was demonstrated with the extracellular protease of B22 with casein as substrate. When extracellular and intracellular protease of the mutant were tested, the absorbance first increased and then decreased. This indicates that the primary attack on casein by proteases of the mutant is different from that of the extracellular protease of the wild type. Fig 6 shows the differences in action on casein of

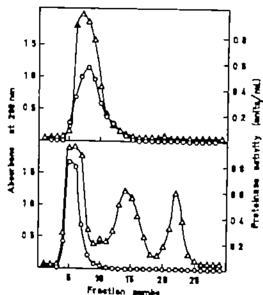


Fig 5 Zone electrophoresis of extracellular protease (upper diagram) and cell extract of B22 1 (lower diagram) The column was packed with Sephadex G-25 in Tris-citrate buffer pH 8.6, ionic strength 0.075. Column dimension 1 cm × 40 cm. The run was performed at 4 C for 5 hours at 500 V and 15 mA. Protease migration was from right to left towards the anode, and the starting point was at position corresponding to fraction 25
Δ Material distribution. ○: Protease activity

the three proteases. Each test sample contained 0.05 protease units per ml determined by the routine protease assay

All the proteases tested hydrolysed the esters ATEE and BAEE at similar rates. The rate of BAEE hydrolysis was, however more than ten times slower than that obtained with trypsin, tested at a concentration which had a similar caseinolytic activity

Dipeptides were not cleaved by the mutant proteases, and the extracellular wild type enzyme is also without effect on such substrates (*Hofsten & Reinhammar* 1965) Poly-L-lysine was hydrolysed by the wild type enzyme but not by any of the proteases of the mutant.

Enzyme stability The wild type extracellular protease is heat stable and not dependent on metal ions for activity or stability In contrast, the protease activity of both culture filtrates and cell extracts of B22 1 was strongly reduced by storage even at room temperature. When the extracellular mutant protease, concentrated by precipitation with ammonium sulphate, was incubated at 55 C for 60 minutes, the activity was reduced by about 50 per cent. The high molecular weight component, P1 from a gel filtration experiment was unaffected by incubation at 55 C for 60 minutes, whereas the same treatment of the other protease component, P2, caused a complete inactivation. Similar tests on the two protease components of cell extracts of B22 1 separated by gel filtration, revealed the same difference in stability

The wild type extracellular protease is completely stable in the presence of EDTA (*Hofsten & Reinhammar* 1965) and no re

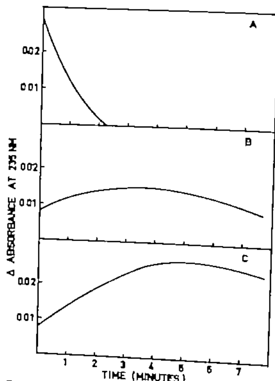


Fig 6 Absorbance changes in a solution of casein caused by extracellular protease of B22 (A) extracellular protease of B22 1 (B) and intracellular protease of B22 1 (C) Substrate: 0.002 per cent casein in 0.02 M potassium phosphate buffer pH 7.4 Each enzyme sample contained 0.05 protease units per ml as determined by the standard protease assay

quirement for divalent cations was reported. Dialysis of the extracellular protease of the mutant against 0.05 M EDTA reduced the activity completely and addition of 0.05 M Ca^{2+} or Mg^{2+} to the dialysed enzyme partly restored the activity. A similar effect was also obtained when cellbound protease was tested. This shows that the extracellular protease of the mutant strain, as well as the intracellular protease are dependent on divalent cations.

Enzyme material corresponding to component P2 (Fig 2) and cell extract of B22 1 were incubated with 1.25 mM di-isopropyl phosphofluoridate (DFP) in 0.1 M Tris-HCl buffer pH 8.0 at 20° C. After one hour 45 per cent of the activity of the extracellular protease and 25 per cent of the activity of the cell bound protease remained. After 20 hours further incubation at 5 C all the activity in both samples was lost. The wild type protease is also inactivated by DFP and all three enzymes are thus of the serine type.

Immunological experiments. Antiserum B prepared against crystallized extracellular protease of strain B22 gave a clear precipitation line, when tested against the culture filtrate of the wild type strain both in gel-diffusion and in immunoelectrophoresis. The purified extracellular protease of the mutant and ten times concentrated culture filtrates did not react with this antiserum.

Antiserum C, prepared against cell extracts of the wild type *Arthrobacter* was used to detect intracellular material in protease preparations from the wild type and the mutant. Purified extracellular protease of the mutant as well as concentrated culture filtrates reacted with this antiserum. On the other hand neither crystallized extracellular protease of the wild type nor concentrated culture filtrates of strain B22 reacted with antiserum C.

These results show that the extracellular protease of the mutant is distinct from the wild type protease, since the former enzyme did not react with antiserum B. The results furthermore show that the mutant releases intracellular antigenic material during growth in contrast to the wild type *Arthrobacter*.

DISCUSSION

The molecular events behind the secretion of proteases are poorly understood, and they are also likely to be different for different groups of bacteria. Most detailed information is available for the genus *Bacillus* where several authors have described mutants producing altered amounts of extracellular protease. *Aronson et al.* (1971) isolated 29 mutants of *B. cereus* which differed in ability to produce protease. They concluded that there are related but distinct catabolic controls for extracellular protease and spore formation. *Hageman & Carlton* (1973) described two protease-deficient mutants of *Bacillus subtilis* one of which lacked a metal-requiring neutral protease and the other had lost an intracellular protease. The latter enzyme was proposed to be involved in intracellular protein turnover and the mutant failed to sporulate normally.

The commonly observed repression of protease synthesis by amino acids has recently been suggested to be due to inhibited transcription of the protease mRNA in Bacillus (*Glenn et al.* 1973).

The secretion of an extracellular enzyme requires the proper functioning of several genes, and changed phenotypes may therefore occur by mutation at several different loci. A mutation in the structural gene of an enzyme may thus result in a protein with changed biochemical properties or its complete disappearance. If regulatory genes are changed, the rate of synthesis of the enzyme may be affected. Finally the secretion mechanism may be influenced by mutations which give rise to changes in the cell envelope.

The proteolytic activity of cultures of the *Arthrobacter* mutant B22 1 grown in the gelatine medium was 10 to 20 per cent of the wild type activity. The amount of enzyme found in the culture supernatant reached a maximum at the end of the exponential growth phase. Prolonged cultivation beyond the exponential phase resulted in a reduction of protease activity. The proteolytic activity of wild type cultures, on the other hand, con-

tinued to increase during the stationary growth phase. As the protease of culture filtrates of strain B22 1 is quite labile at 30 C, the mutant probably also releases protease during the stationary growth phase, because the activity would otherwise decrease more rapidly than observed.

The experimental results, summarized in Table 1 show that the heat labile extracellular protease of the mutant is unrelated to the wild type extracellular protease because a) The elution volumes in gel filtration experiments are different. b) The charge of the wild type and the mutant proteases is reversed at pH 8.6. c) The wild type enzyme is insensitive to EDTA, whereas the mutant protease requires divalent cations for activity and stability d) Only the wild type enzyme cleaves poly-L-lysine. e) The mutant does not form material which reacts with antiserum against the extracellular protease of the wild type strain.

Our conclusion is that the protease in culture fluids of the mutant and the wild type extracellular enzyme are coded by different genes, and that the mutant is incapable of forming normal extracellular protease.

Several similarities between the cell bound and the extracellular proteases of the mutant suggest that they are identical but unfortunately because of their lability they could not be purified.

A model for the formation and secretion of extracellular protease in *Bacillus amyloliquefaciens* has recently been proposed (*Both et al.* 1972) According to this model, the site of translation of protease messenger RNA in the cell and the cell structure responsible for the secretion of the extracellular protease are the same a ribosome-cell membrane complex. Mutational changes affecting such a translation-extrusion structure could give phenotypes which are indistinguishable from phenotypes resulting from mutations in the regulatory or structural genes of the extracellular protease.

The mutant described in this paper differs from the wild type in two main respects (i) Loss of ability to produce an extracellular protease in active or immunologically recognizable form (ii) Release of intracellular protease through the cell membrane.

Whether these two phenotypic changes are caused by a single mutation affecting both characters or by two mutations cannot be decided.

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INFECTION OF *ARVICOLA TERRESTRIS* (VOLE RAT) WITH *M TUBERCULOSIS* AND *M BOVIS*

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Arvicola terrestris (vole rats) which belong to the vole family have not been used previously for infection experiments with tubercle bacilli. In the present study the susceptibility of these animals to *M tuberculosis* and *M bovis* has been examined by intraperitoneal, subcutaneous and intravenous injection of varying doses of finely dispersed bacterial suspensions. Characteristic for the infection with *M bovis* is a strong multiplication of the bacteria and the development of macroscopically visible caseous processes in liver spleen and lungs, and in the enlarged lymph glands. The organs—in particular spleen, lungs and lymph glands—contain enormous numbers of tubercle bacilli. Doses as small as a few viable units are able to induce lethal infection. In contrast, the virulence of *M tuberculosis* is slight. The bacteria multiply sparsely and cause insignificant macroscopical changes in the organs. The course is markedly chronic unless use is made of giant doses administered intravenously. The susceptibility of vole rats to the two bacterial species resembles that of rabbits, and thus these animals can be used for differentiation purposes.

The susceptibility of voles to *M tuberculosis* and *M bovis* is quite different from that of murids. While the white mouse is resistant to both bacterial species, the vole is resistant to *M tuberculosis* but extremely sensitive to *M bovis*. Of the vole family the common vole (*Microtus arvalis*) (*Koch* 1912 *Jespersen* to be published) and the field vole (*Microtus agrestis*) (*Wells* 1938 *Griffith* 1937 1939 1941) have been used for infection experiments with tubercle bacilli, and also the red mouse (*Clethrionomys g glareolus Schreb*) (*Jespersen* 1954 *Hauduroy & Bize* 1957 *Bize* 1961) The present work deals with infection with *M tuberculosis* and *M bovis* in a fourth member of the vole family viz. *Arvicola terrestris* (vole rat)

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MATERIAL AND METHODS

Experimental animals: The experiment comprised 33 captured vole rats which before infection were placed individually in guinea pig cages at random.

Bacterial strains Before the experiment, *M bovis* strain E6884B was inoculated into a rabbit and the *M tuberculosis* strain E10883H into a guinea pig. The animals were allowed to die spontaneously and a suitable organ was used for culture of the strains on Löwenstein-Jensen medium followed by four subcultures on Dubos fluid medium with Tween.

Bacterial suspensions for infection About 10 ml culture of each strain (10 days old on day of experiment) was shaken until homogeneous macroscopically and then exposed to ultrasonic treatment for 10 minutes at an intensity of 0.58 watts/cm². After treatment, the two suspensions contained about 90 per cent single bacteria, about 10 per cent

Animal	*M. bovis*			*M. tuberculosis*		
	Viable units	Survival time (days)	Degree of infect.	Viable units	Survival time (days)	Degree of infect.
	7 i.p.	k 75	II	8 i.p.	k 75	V
Guinea	7 i.p.	k 75	V	8 i.p.	k 75	V
pig	73 i.p.	k 75	V	76 i.p.	k 75	V
	73 i.p.	k 75	V	76 i.p.	k 75	V
Rabbit	7.3×10^4 i.v.	†24	V	7.6×10^4 i.v.	k 101	I
	7.3×10^4 i.v.	†23	V	7.6×10^4 i.v.	k 101	I

k = killed, † = died spontaneously

units of to 5 bacteria, and no units of more than 5 bacteria. The content of viable units in the suspensions was determined by inoculating suitable dilutions on Dubos oleic acid albumin agar plates. The colony count showed that each animal injected with 0.2 ml of dilution 10^{-8} had been given 8 bacteria of *M. tuberculosis* and 7 bacteria of *M. bovis*.

The doses used for subcutaneous infection with *M. tuberculosis* were 1 mg and 10 mg semi-dried weight of Besredka culture. Inoculation of suitable dilutions showed that 1 mg culture contained 9.8 $\times 10^4$ viable units.

Virulence determination of strains. At the same time as the strains were injected into vole rats, virulence determination was made on rabbits and guinea pigs (see table above).

Experimental. The animals were divided into three groups to be infected subcutaneously, intraperitoneally or intravenously. Each group was again divided into two subgroups for infection with *M. tuberculosis* and *M. bovis* respectively. A pilot study had shown that vole rats resembled the other voles in respect of their susceptibility to the two bacterial species, and therefore large doses of *M. tuberculosis* and very small doses of *M. bovis* were used for infection. The subcutaneous injections were made in the right inguinal region and the intravenous injections into a tail vein. The amount administered was 0.2 ml. After infection the animals were allowed to live until they died spontaneously. At autopsy smears were made from liver, spleen, kidneys, lungs and lymph glands, and stained by the Ziehl-Neelsen method. In animals where no tuberculous processes were observed macroscopically or where there were only small processes, culture was made from the organs.

RESULTS

Intraperitoneal infection with M. bovis *(Table 1).* Quite small doses of *M. bovis* caused progressive fatal tuberculosis in the vole rats. Animal No. 45, infected with 730 viable units, survived only 66 days. Autopsy showed no tubercles in liver, kidneys and spleen, but the spleen was somewhat enlarged. There was a small number of just visible grey tubercles in the lungs. Smears from the liver, and particularly from spleen and lungs, showed enormous quantities of tubercle bacilli. The animals infected with 73 viable units survived 140 and 235 days, while those infected with 7 viable units lived 210 and 244 days. The autopsy findings were almost identical in the individual animals and can thus be described together. In two animals there was a caseous abscess at the site of injection. The omentum was retracted, thickened and yellow, or contained yellow tubercles up to the size of hempseed. The lymph glands (portal, mesenteric, lumbar and tracheal glands) were the size of rice grains to peas, and were completely caseous. The spleen, and occasionally also the liver, contained from just visible to millet-sized grey and yellow tubercles and the spleen was somewhat enlarged. There were no tubercles in the kidneys. The lungs were enlarged to twice their normal size and contained large numbers of grey or yellow tubercles up to the size of hempseed. There were numerous or innumerable tubercle bacilli in the smears from lymph glands and lungs. The number was somewhat smaller in spleen, liver and kidneys.

Intraperitoneal infection with M. tuberculosis *(Table 2).* With the large doses used

in this study *viz* 7.6 × 10⁶ and 7.6 × 10⁴ the *M. tuberculosis* infection was lethal but had an extremely chronic course. With the exception of vole rat No. 22, which died of intercurrent disease on the 114th day (culture from liver spleen and lungs negative) the remaining animals survived for 1 to 2 years. Apart from the processes in the lungs the macroscopical changes were insignificant. In two of the animals there were no tubercles in the omentum, and in the third there was a single, non-caseous tubercle the size of a pinhead. Generally the lymph glands were not enlarged in a few cases they were millet sized but were never caseous. There was no enlargement of the spleen and no tubercles in spleen and kidneys. Neither were there tubercles in the liver In the liver of animal No. 19 there was a pea-sized abscess with caseous content. The lungs showed nu[illegible]ous just visible to pinhead-sized grey [illegible]rcles. Microscopy of smears from the [illegible]s revealed innumerable tubercle bacilli in [illegible] animals. Only a few or no bacteria were [illegible]d in liver spleen, kidneys and lymph [illegible]ds of the animal that died on the 369th [illegible] but there were many bacteria in the two [illegible]mals that survived the longest.

[S]ubcutaneous infection with M. bovis *[(T]able 3*) The doses used, *viz.* 73 and 7 [via]ble units, provoked progressive fatal tuber[cul]osis in all the animals. The survival times [var]ied from 241 to 305 days. In the animals [inf]ected with the largest doses, a subfascial [or] intramuscular abscess was found in the [rig]ht inguen. The lymph glands were generally very enlarged and caseous in all the animals. This applied not only to the regional [gla]nds, but also to the mesenteric, portal and [tra]cheal glands, and possibly also to the peri[ph]eric glands such as the submaxillary gland. [T]he glands were generally the size of peas, [bu]t the regional lumbar gland could be as [lar]ge as a date kernel. They were completely [cas]eous and contained numerous or innumerable tubercle bacilli. In the animal that [su]rvived the longest, the glands were calcified [a]nd almost stony The liver spleen and lungs [co]ntained tubercles that were generally caseous. The spleen and lungs were enlarged. There were no tubercles in the kidneys. The number of tubercle bacilli was greatest in the lungs, somewhat less in spleen and liver and lowest in the kidneys.

Subcutaneous infection with M. tuberculosis *(Table 4)* The virulence of *M. tuberculosis* was slight. In vole rats Nos. 50 and 48 infected with 10⁸ and 10⁷ viable units, respectively except for an abscess at the site of injection, there were no macroscopical signs of tuberculous processes in lymph glands, liver spleen, kidneys and lungs. Tubercle bacilli could be demonstrated by microscopy of the abscess. Culture revealed a few viable units in the lymph glands, liver and lungs of vole rat No. 50 In animal No. 48, two viable units were found by culture from the lumbar gland, while culture from liver spleen and lungs gave no growth. These animals cannot have died of a tuberculous infection. Nos. 49 and 47 presumably died of their tuberculous lung processes, but also in these animals the numbers of bacteria in lymph glands, liver spleen and kidneys were small. Even in the affected lungs there were only few bacteria. Regressive processes were prominent. This applies both to the local abscess and the lymph glands and in one vole rat to tubercles in the lungs.

Intravenous infection with M. bovis *(Table 5)* Doses of 730 73 and 7 viable units produced a fatal infection, with survival times varying from 148 to 268 days.

In vole rat No. 46 infected with 730 viable units, there were tubercles in the spleen but otherwise no macroscopical signs of tuberculosis. However despite this, smears from the organs contained numerous or innumerable tubercle bacilli, with particularly large numbers in the lungs.

The autopsy findings in the remaining animals varied only slightly from animal to animal. The liver spleen and lungs contained tubercles which were generally caseous. The spleen and lungs were enlarged. There were no tubercles in the kidneys. The lumbar, mesenteric, portal and sometimes the tracheal glands were enlarged (the size of a pea to

TABLE 1 *Intraperitoneal Infection of Val*

Animal number	Strain	Dose	No. of viable units	Survival time (days)	Omentum	Lymph	
						Portal	Mesenteric
45	E6884B	10^{-4}	7.3×10^{2}	†66	—	—	—
37	–	10^{-5}	7.3×10	†140	—	+ (∞)	+ (∞)
38	–	10^{-5}	7.3×10	†235	+	+ (+ + +)	+ (+ + +)
34	–	10^{-6}	7	†210	+	+ (+ + +)	+ (+ + +)
33	–	10^{-6}	7	†244	+	+ (+ + +)	+ (∞)

+ or — under Lymph glands indicate whether or not these were caseous.
+ or — under "Omentum, Liver Spleen, Kidney Suprarenal gland and Lung" indicate whether or not these organs contained any macroscopical tuberculosis processes.
Symbols in brackets indicate the bacillary findings by microscopy of smears, expressed as + to ∞.
† = Died spontaneously

TABLE 2. *Intraperitoneal Infection of Val*

Animal number	Strain	Dose	No. of viable units	Survival time (days)	Omentum	Lymph	
						Portal	Mesenteric
22	E10883H	10^{0}	7.6×10^{6}	†114	—	—	—
21	–	10^{0}	7.6×10^{6}	†634	+	—	—
20	–	10^{-3}	7.6×10^{3}	†369	—	—	—
19	–	10^{-3}	7.6×10^{3}	†712	—	—	—

Symbols in brackets indicate the bacillary findings by microscopy of smears, expressed as + to ∞ or the *number of viable units (vu)*
* Died of intercurrent disease.
† = Died spontaneously

TABLE 3 *Subcutaneous Infection of Val*

Animal number	Strain	Dose	No. of viable units	Survival time (days)	Abscess	Lymph	
						Subcut.	Lumbar
40	E6884B	10^{-5}	7.3×10	†241	+ (+ + +)	—	+ (∞)
39	–	10^{-5}	7.3×10	†505	+ (+)	+ (—)	+ (+ + +)
35	–	10^{-6}	7	†265	—	—	+ (∞)
36	–	10^{-6}	7	†273	—	—	+ (+ + +)

For symbols see Table 1

that of a date kernel) and were transformed into a yellow caseous mass. The number of bacteria was very large in all organs, even in the kidneys.

Intravenous infection with M *tuberculosis (Table 6)* The animals infected with 7.6×10^{6} units died on the 168th and 170th day as the result of the infection. Even though there were no macroscopical signs of tuberculosis, culture from lungs and liver (and spleen in one of the animals) gave growth of innumerable viable units. The animals infected with 7.6×10^{4} and 7.6×10^{6} and one of those infected with 76 viable units (survived 913 days) did not die of tuberculosis. Macroscopically there were no tuber

...ts (Arvicola terrestris) with M. bovis

...glands Lumbar	Tracheal	Liver	Spleen	Kidney	Suprarenal gland	Lung
—	—	—(∞)	—(∞)	—	—	+(∞)
+(∞)	—	—(+++)	+(+++)	—	—	+(∞)
+(+++)	+(++)	+(+)	+(+)	—(—)	—	+(+++)
+(+++)	+(∞)	—(+)	+(+)	—(+)	—	+(∞)
+(∞)	+(+++)	+(+++)	+(∞)	—(++)	—	+(∞)

(Arvicola terrestris) with M. tuberculosis

...ds ...umbar	Tracheal	Culture of pooled lymph glands	Liver	Spleen	Kidney	Suprarenal gland	Lung
—	—		—(0 vu)	—(0 vu)	—	—	—(0 vu)
—	—	(∞vu)	—(+)	—(+)	—(∞)	—	—(∞)
—	—	(0 vu)	—(+)	—(+)	—(—)	—	+(∞)
—	—	(∞vu)	+(+)	—(+++)	—(+++)	—	+(∞)

...ts (Arvicola terrestris) with M. bovis

...nds Mesent.	Portal	Tracheal	Liver	Spleen	Kidney	Suprarenal gland	Lung
+(∞)	+(+++)	—	—(+)	+(+++)	—(—)	—	+(∞)
—	+(+)	—	+(+)	—(+)	—(+)	—(—)	+(∞)
+(+++)	+(++)	+(∞)	+(++)	—(+++)	—(+)	—	+(∞)
+(+)	+(+++)	+(—)	+(—)	+(++)	—(—)	—	+(+)

culous processes, except for a calcified tubercle in the liver and a regressive tubercle in the lung of the animal that survived the longest. Culture from the organs was generally negative, and in the few cases where there was growth, the number of colonies was very small. However one animal infected with 76 viable units and both animals infected with 8 viable units, died of pulmonary tuberculosis. A description of the autopsy findings for the individual animals provides a good illustration of the chronic course of the disease.

Vole rat No. 26 (76 viable units) died on the 386th day. No definite tubercles in liver spleen, kidneys or lungs. Spleen not enlarged.

TABLE 4 *Subcutaneous Infection of V[...]*

Animal number	Strain	Dose	No. of viable units	Survival time (days)	Abscess	Lym[...]		
						Subc.	Lumbar	Mesen[...]
49	E10883H	10 mg	9.8×10^{7}	†528	+ (+)	—	—	—
50	–	10 mg	9.8×10^{7}	†586	+ (+)	—	—	—
47	–	1 mg	9.8×10^{6}	†441	—	—	—	—
48	–	1 mg	9.8×10^{6}	†452	+ (+ + +)	—	—	—

For symbols see Table 2.

TABLE 5 *Intravenous Infection of Vo[...]*

Animal number	Strain	Dose	No. of viable units	Survival time (days)	Lymp[...]	
					Portal	Mesent.
46	E6884B	10^{-}	7.3×10^{3}	†148	—	—
51	–	10^{-4}	7.3×10^{2}	†134	+ (+ + +)	+ (∞)
44	–	10^{-5}	7.3×10	†152	+ (+ + +)	+ (+ + +)
43	–	10^{-5}	7.3×10	†268	+ (∞)	+ (∞)
41	–	10^{-6}	7	†197	+ (+ + +)	+ (∞)
42	–	10^{-6}	7	†244	+ (+ + +)	+ (+ + +)

For symbols see Table 1

TABLE 6. *Intravenous Infection of Vol[...]*

Animal number	Strain	Dose	No. of viable units	Survival time (days)	Portal	Mesent.	Lymph[...] Lumbar
32	E10883H	10^{0}	7.6×10^{5}	†168	—	—	—
31	–	10^{0}	7.6×10^{5}	†170	—	—	—
29	–	10^{-2}	7.6×10^{4}	†201	—	—	—
30	–	10^{-2}	7.6×10^{4}	†213	—	—	—
27	–	10^{-}	7.6×10^{3}	†109	—	—	—
28	–	10^{-}	7.6×10^{3}	†822	—	—	—
26	–	10^{-5}	7.6×10	†386	—	—	—
25	–	10^{-5}	7.6×10	†913	—	—	—
23	–	10^{-6}	8	†579	—	—	—
24	–	10	8	†1046	—	—	—(+)

For symbols see Table 2.

Mesenteric glands slightly enlarged but not caseous, and other glands normal. Smears from lungs showed innumerable tubercle bacilli and from liver spleen and kidneys a few to numerous bacteria.

Vole rat No. 23 (8 viable units) died on the 579th day No definite tubercles in liver spleen, kidneys and lungs. Spleen not enlarged. Mesenteric and tracheal glands slightly enlarged but not caseous. Smears from lungs showed innumerable tubercle bacilli, from kidneys none, and from spleen a few bacteria. There were a few viable units in liver and lymph glands

Vole rat No 24 (8 viable units) died on the 1046th day No tubercles in liver spleen

…ats (Arvicola terrestris) with M. tuberculosis

…lands		Cult. of pooled lymph glands	Liver	Spleen	Kidney	Suprarenal gland	Lung
Port.	Trach.						
—	—	(9 vu)	—(—)	—(—)	—(+)	—	+(+)
—	—	(40 vu)	—(1 vu)	—(0 vu)	—(—)	—	—(77 vu)
—	—	(7 vu)	—(0 vu)	—(0 vu)	—(—)	—	+(+)
—	—	(2 vu)	—(0 vu)	—(0 vu)	—(—)	—	—(0 vu)

…ats (Arvicola terrestris) with M. bovis

…ands		Liver	Spleen	Kidney	Suprarenal gland	Lung
Lumbar	Tracheal					
—	—	—(+++)	+(∞)	—(+++)	—	—(∞)
+(+++)	—	+(++)	+(+++)	—	—	+(∞)
+(∞)	—	+(++)	+(+++)	—(+++)	—	+(∞)
+(∞)	—(+)	—(+)	+(+++)	—(+)	—	+(+++)
+(+++)	+(∞)	+(+++)	+(∞)	—(++)	—	+(∞)
+(+++)	—	+(++)	+(∞)	—(+++)	—	+(∞)

Rats (Arvicola terrestris) with M. tuberculosis

…glands	Cult. of pooled lymph glands	Liver	Spleen	Kidney	Suprarenal gland	Lung
Tracheal						
—		—(∞ vu)	—(∞ vu)	—	—	—(∞ vu)
—		—(300 vu)	—(26 vu)	—	—	—(∞ vu)
—	(0 vu)	—(0 vu)	—(0 vu)	—(—)	—	—(0 vu)
—	(0 vu)	—(0 vu)	—(0 vu)	—(—)	—	—(1 vu)
—		—(—)	—(—)	—	—	—(+)
—	(30 vu)	—(0 vu)	—(2 vu)	—(—)	—	—(1 vu)
—	(219 vu)	—(+)	—(++)	—(+++)	—	—(∞)
—	(13 vu)	+(0 vu)	—(6 vu)	—(—)	—	+(1 vu)
—	(75 vu)	—(7 vu)	—(+)	—(—)	—	—(∞)
—(+)		—(600 vu)	—(+)	—(—)	—	+(+++)

and kidneys. Spleen slightly enlarged. Lungs very enlarged and almost completely transformed into a uniform greyish-red solid mass consisting of confluent tubercles. Lumbar and tracheal glands slightly enlarged grey and semi-translucent. Mesenteric and portal glands millet-sized, almost transparent. On the back of the sternum there was a solid, yellowish gland the size of hempseed. Smear from lungs showed numerous tubercle bacilli, from the spleen a few from the kidneys none, and from the lymph glands a few bacteria. There were 600 viable units in the liver

DISCUSSION

The vole family which comprises *Microtus arvalis* (common vole) *Microtus agrestis* (field vole), *Clethrionomys glareolus* (red mouse) and *Arvicola terrestris* (vole rat) distinguishes itself both in appearance and mode of living from murids (house mouse, white mouse, rat, etc.)

Robert Koch found in 1884 that *Microtus arvalis** was much more susceptible to tubercle bacilli than the white mouse. He did not observe any difference in the effect of strains isolated from various tuberculous diseases in man and a strain isolated from a case of bovine tuberculosis. In contrast to *Koch's* findings, the writer has observed in an experiment as yet not published that *Microtus arvalis* is highly susceptible to *M bovis* and strongly resistant to *M tuberculosis* *Wells* (1938) and *Griffith* (1937 1939 1941) examined the susceptibility of the two bacterial species in *Microtus agrestis* *Wells* concluded from his experiment that in order to provoke progressive tuberculosis, it is necessary to inject 1 mg of *M tuberculosis* intraperitoneally and at most 10^{-8} mg *M bovis* thus indicating that *Microtus agrestis* is at least a hundred thousand times more susceptible to *M bovis* than to *M tuberculosis*. *Griffith* using subcutaneous infection arrived at a similar conclusion, and furthermore demonstrated that *Microtus agrestis* is just as sensitive to *M bovis* as the guinea pig Both workers recommended the use of field mice for differentiation of the two bacterial species.

The same difference between *M bovis* and *M tuberculosis* infection has been demonstrated in red mice (*Jespersen* 1954) and his results have been confirmed by *Hauduroy* & *Buse* (1957) and *Buse* (1961)

Vole rats also possess the highest susceptibility to *M bovis* The lowest dose (7 viable units) regularly produced a progressive lethal infection. This was the case whether the bacteria were injected intravenously intraperitoneally or subcutaneously The survival times were shortest in the animals infected intravenously and longest in those infected subcutaneously but the difference does not seem to be marked. Dosage is also of relevance for the course. As an example, it can be stated that in the pilot study two animals injected intravenously with 2.7×10^5 viable units died after 25 and 26 days, while in the present study two injected with 7 viable units died after 197 and 244 days.

The *M bovis* infection was characterized by an enormous multiplication of bacteria and development of caseous processes in liver spleen, lungs and lymph glands containing numerous or innumerable tubercle bacilli. The spleen, lungs and lymph glands were enlarged, and it was particularly in those organs that the number of bacteria reached enormous heights.

The course of the *M tuberculosis* infection in vole rats was markedly chronic. Intravenous injection with the lowest dose of *M tuberculosis* caused the animals to die after 579 and 1046 days. The corresponding survival times after *M bovis* infection were 197 and 244 days.

The course is very dependent on the route of infection. 7.6×10^4 viable units injected intravenously caused death after 168 and 170 days, and after 634 days when given intraperitoneally Two animals injected subcutaneously with a giant dose of 10^7 viable units did not die until the 528th and 586th days. There were only few bacteria in the organs, and only the one animal could be assumed to have died from tuberculosis.

Dosage is also of significance. It is evident from Table 6 that the survival times are prolonged as the dose decreases.

Multiplication of *M tuberculosis* presumably takes place a short time after the injection, after which it ceases and the organism gradually eliminates the majority of the bacteria. No growth was found by culture from liver spleen, lungs and lymph glands in animals injected intravenously with 7.6×10^4 viable units, except for one animal where one viable unit was found in a lung A few

* Called by *Koch* *Arvicola arvalis* a name previously used for the species.

bacteria survive, and the same applies in the case of infection with minimal doses. When for some reason or other the resistance of the animal is reduced, the bacteria in the lungs begin to multiply and cause a tuberculous disease which leads to death (Table 6)

While severe caseous processes developed in the organs after infection with *M bovis* the macroscopical processes were inconsider able after infection with *M tuberculosis.* Tubercles in liver spleen and kidneys were never seen, and the spleen was not enlarged. The lymph glands were either not enlarged at all or only very slightly and they never be came caseous. Tubercles in the lungs were found only in the final stages in animals that survived for 1 to 3 years.

Thus, in their susceptibility to the two bacterial species, vole rats resemble rabbits and can therefore be used for differentiation. The difference in the effect of *M tubercu losis* and *M bovis* was at its maximum when the bacterial suspension was injected subcu taneously For practical reasons (in order to avoid the development of an abscess at the site of injection) intravenous injection is preferable. A dose of about 10^{4} viable units would presumably be suitable. 2.7×10^{4} viable units of *M bovis* killed the animals after 25 and 26 days, and 7.6×10^{4} viable units of *M tuberculosis* after 168 and 170 days. Differentiation can be made both on the basis of the survival times and on the macro-scopical processes.

In order that animals can be used for experimental purposes, it is a condition that they should be able to breed in captivity Ex periments have shown that this is the case with vole rats.

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INDUCTION OF SV40-TUMOUR IMMUNITY BY SV40-TRANSFORMED CELLS IN DIFFUSION CHAMBERS

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The level of transplantation immunity against syngeneic SV40 tumour cells was studied in CBA mice after implantation of diffusion chambers containing SV40-transformed cells. The immunization effect was dependent on the duration of chamber deposition, the site of implantation and the pore size of the chambers. 10^5 irradiated ME1 C9-V15 cells in 0 45 µm chambers deposited for 1 or 3 days subcutaneously induced a significant level of immunity as shown by challenge 3 weeks later (Sensitivity index, SI = $10^{1.4}$ and $10^{1.9}$) After 7 or more than 21 days of deposition the level of immunity at 3 weeks was increased (SI = $10^{2.7}$ and $10^{2.8}$) and almost equivalent to that produced by inoculated cells (SI = $10^{2.8}$ and $10^{3.0}$) Ten weeks after immunization with cells in diffusion chambers, the immunity had decreased and was weaker than that induced by directly inoculated cells, both when irradiated and non-irradiated cells were used for immunization. Cells in chambers with 0.1 µm pores were less immunizing than cells in chambers with 0.45 µm pores. Normal spleen cells added to irradiated tumour cells in chambers with 0.1 µm pores caused a significant level of immunity where the tumour cells alone did not. The immunization effect of syngeneic as well as of xenogeneic SV40-transformed cells was weaker after intraperitoneal than after subcutaneous inoculation and cells enclosed in diffusion chambers deposited intraperitoneally did not immunize at all.

In a recent investigation of the development of SV40 tumour immunity in CBA mice (*Stillström* 1974) it was shown that the level of early immunity was dependent on the immunization dose of proliferative or non-proliferative cells, but the early tumour formation was not found to have any influence. When the tumours later reached a certain size, however the level of immunity began to decrease. The importance of the contact between animal and antigenic tumour cells and of the site of the contact was now studied in the same tumour system with cells enclosed in millipore diffusion chambers of different porosities, deposited subcutaneously or intraperitoneally Of special concern was the question whether immunity could be induced in a short time with cells in diffusion chambers *in vivo* and whether the withdrawal of the chambers, like the resection of tumours, did not reduce the level of immunity Furthermore, it was of interest to know whether cells in chambers deposited intraperitoneally were able to induce immunity If so, immunoselection could be a determining factor for the oncogenic potential of the enclosed cells. In an attempt to induce immunity with the transplantation antigen *in situ* normal spleen cells were added to irradiated tumour cells in diffusion chambers.

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MATERIAL AND METHODS

Animals

Male CBA mice were obtained from the Department of Genetics, University of Stocholm, Sweden.

Cells

SV40-transformed syngeneic and xenogeneic cells were used for immunization, viz. the cloned mouse embryo cell line ME1 C9 and the highly oncogenic ME1 C9 derived tumour line ME1 C9-V15 (*Wesslén* 1970 *Stillström* 1975) and the rat tumour line R92 (*Diderholm et al.* 1966) The ME1 C9-V15 line and another syngeneic SV40-transformed tumour line, ME1 V46 derived from non-cloned mouse embryo cells, were used for challenge. The culture conditions, the treatment of the cells and the immunization and challenge procedures have been described previously (*Stillström* 1975)

Immunity

The immune state of an animal group was expressed as the sensitivity index (SI) By SI is meant the quotient of the tumour producing doses (TPD_{50}) of challenge cells in immunized and non-immunized animals. The immunizing dose (ImD_{50}) is defined as that dose of cells which protects 50 per cent of animals from tumour formation after a fixed challenge dose (*Stillström* 1974) Calculations of TPD_{50} and ImD_{50} were made according to *Kärber* (1931) The animals were irradiated (300 R) the day before challenge.

Diffusion Chamber T knique

Millipore filters (Millipore, Bedford, Mass., U.S.A.) with a porosity of 0.45 μm and a thickness of 100 μm (TWWP) and with a porosity of 0.1 μm and a thickness of 140 μm (VCWP) were glued (MF Cement No. 1) to plexiglass rings 2 mm high and with an external diameter of 14 mm. Half-completed chambers were filled with 0.05 ml cell suspension and sealed with the top filter The animals were anaesthetized with ether the abdominal skin was shaved and cleaned with alcohol and a lateral incision was made through the skin. For subcutaneous chamber deposition, a pocket was formed by blunt dissection ventrally and for intraperitoneal deposition an opening was cut in the peritoneum with a pair of sharp scissors. The wounds were closed by silk sutures. The chambers never eroded from the intraperitoneal position, but were always rejected if left subcutaneously for several weeks. The chambers were removed through a contralateral incision and released from adherent tissues. The operation mortality was negligible. Before insertion and after removal from the animals, the chambers were kept in culture medium and the medium was then cultivated to show the presence of leaking tumour cells. Tumour cells and cells originating from the host could be differentiated by immunofluorescence and by their growth characteristics. The extent of leakage was determined more quantitatively by comparing the tumour incidences of graded doses of non-irradiated cells, either inoculated subcutaneously or enclosed in chambers deposited subcutaneously Tumours appearing at the sites of inoculation and the sites of chamber implantation were recorded weekly and the TPD_{50}s estimated. The leakage quotient was calculated as the ratio of the TPD_{50} of directly inoculated cells and the TPD_{50} of chamber enclosed cells expressed by leakage. A leakage quotient was also calculated on the immunizing doses (ImD_{50}) of the cells.

RESULTS

The Level of Immunity after Subcutaneous Inoculation

Six to seven-week-old animals were used for immunization in order to avoid large age-dependent differences in tumour resistance between animals challenged after different times. The animals were inoculated with 10^6 irradiated ME1 C9-V15 cells subcutaneously and challenged with graded doses of the same cells after 3 7 or 21 days. Animals challenged 3 days after immunization were not irradiated before challenge and animals challenged after 7 or 21 days were either irradiated (300 R) the day before challenge or not irradiated. As early as 3 days after immunization, the animals were slightly immune ($SI = 10^{1.9}$ Table 1) and 7 days after immunization, irradiated animals resisted 10,000 times as many tumour cells as did the control animals (TPD_{50}s $10^{7.5}$ and $10^{3.5}$ cells, respectively $SI = 10^{4.0}$) Twenty one days after immunization, the high level of immunity was maintained ($SI \geq 10^{3.5}$ in irradiated animals, Table 1)

Three to four-week-old animals were immunized as above and challenged with ME1 C9-V15 cells after 3 or 10 weeks. After 3 weeks the SI was $10^{2.4-4.4}$ and after 10 weeks 10^3 (Table 2)

After inoculation of 10^6 irradiated ME1 C9-V15 cells, immunization was thus completed in 7 days and the level of immunity

TABLE 1 *Immune State of Animals 3,7 and 21 Days after Subcutaneous Inoculation of 10^8 Irradiated ME1 C9-V15 Cells and 21 days after Intraperitoneal Deposition / Diffusion Chambers Containing the Same Immunization Dose as Determined by Graded Challenge with ME1 C9-V15 Cells*

Immunization)			Challenge						
			Immunized animals			Non-immunized animals			
Mode	Irradiation of animals[a])	Time post immunization (days)	Dose range	No. of animals	^{10}log TPD_{50}	Dose range	No. of animals	^{10}log TPD_{50}	^{10}log SI[3])
directly subcutaneously		3	10^2–10^7	18	6.2	10^2–10^6	15	5.2	1.0
		7	10^2–10^7	18	≥7.5	10^2–10^6	15	5.5	≥2.0
	+	7	10^2–10^7	18	7.2	10^2–10^6	10	3.2	4.0
		21	10^2–10^7	14	7.2	10^2–10^6	12	5.8	1.4
	+	21	10^2–10^7	13	≥7.5	10^2–10^6	12	4.5[5])	≥3.0
via 0.45 μm diffusion chambers, 91 days intraperitoneally[4])	+	21	10^6–10^8	12	4.2			4.5[5])	-0.3

) ME1 C9-V15 cells used for immunization were X-irradiated (10,000 R) immediately before inoculation or deposition in diffusion chambers.
[a]) X-irradiation (500R) of the animals, the day before challenge.
[3]) Sensitivity index.
) The chambers were implanted 3 weeks before challenge and removed 10 weeks after
[5]) Identical control.

was maintained on a high level for at least 10 weeks.

The Level of Immunity after Immunization with Cells in Diffusion Chambers Deposited Subcutaneously

Three- to four week-old animals were immunized with 10^6 irradiated ME1 C9-V15 cells contained in diffusion chambers (pore size 0.45 µm) deposited subcutaneously for 1 3 5 7 and 22—28 days and challenged with graded doses of ME1 C9-V15 cells on the 21st—24th after implantation of the chambers. One or 3 days were sufficient for immunity to be induced (SI = $10^{1.0}$ and $10^{1.9}$ respectively Table 2) and deposition for 7 days or 22—28 days gave an increased protection (SI = 10^{2} and $10^{2.6}$) equivalent to that induced by inoculated cells (SI = $10^{2.8}$ and $10^{2.4}$ Table 2) After ten weeks the level of immunity induced by irradiated or non-irradiated cells in diffusion chambers deposited subcutaneously for 7 days was 10–100 times lower than that achieved with directly inoculated cells (TPD_{50} $10^{3.1-4.1}$ vs. $10^{4.1-4.6}$ Table 2)

10^6 irradiated ME1 C9-V15 cells deposited subcutaneously for 5 days in diffusion chambers with a pore size of 0.1 µm did not induce immunity (SI = $10^{-0.5}$) but when the time of deposition was extended to 7 days or when 2 × 10^6 syngeneic spleen cells from non-immunized animals were added to the tumour cells in the chambers a state of immunity was achieved (SI = $10^{1.3}$ and $10^{1.3}$ Table 2)

Estimation of the Leakage of Cells from Diffusion Chambers Subcutaneously

Graded doses of non-irradiated ME1 C9-V15 cells were inoculated subcutaneously in one group of animals and were enclosed in diffusion chambers with 0.45 µm pores and deposited for 7 days subcutaneously in another The TPD_{50} of the first group was $10^{1.6}$ cells and that of the second group $10^{4.1}$ cells after 10 weeks of observation (Table 3) Two progressively growing and 2 regressive tumours had then appeared in 13 animals at the sites of chamber implantation. The leakage quotient was $10^{-2.0}$ which indicated that on the average the leakage was below 1 per cent of the number of the cells enclosed. Ten weeks after immunization, animals surviving tumourfree were irradiated (300 R) and challenged the following day with 10^4 ME1 C9-V15 cells in order to determine the immunizing dose of the primarily administered cells. The ImD_{50} of directly inoculated cells was $10^{1.8}$ and that of chamber enclosed cells $10^{3.8}$ cells, making a leakage quotient of $10^{-2.0}$ In a similar experiment, ME1 C9-V15 cells enclosed in diffusion chambers with 0.1 µm pores were deposited subcutaneously for 3 days. After 3 weeks, no tumours caused by leakage of cells had appeared in 25 animals exposed to 10^3–10^6 non-irradiated cells within chambers, whereas directly inoculated cells showed a TPD_{50} of $10^{1.3}$ cells. The leakage quotient was thus at most $10^{-4.5}$ To determine the ImD_{50}s of the cells in direct continuous contact with the animal tissues and of the cells in indirect contact for 3 days via 0.1 µm pore filters, the animals were challenged with 10^4 ME1 C9-V15 cells. The ImD_{50}s were $10^{2.2}$ and $10^{6.3}$ cells, respectively and the leakage quotient calculated on these figures was thus $10^{-4.3}$

Comparison between Immunization Effects Induced Subcutaneously and Intraperitoneally

Six to seven-week-old animals were either immunized with 10^6 irradiated ME1 C9-V15 cells deposited in 0.45 µm pore diffusion chambers intraperitoneally for 91 days or directly inoculated subcutaneously After 3 weeks, the animals were challenged with graded doses of ME1 C9-V15 cells. The attempt to induce immunity by cells in intraperitoneal diffusion chambers failed (SI = $10^{-0.3}$ Table 1 and Figure 1) but subcutaneous inoculation resulted in a strong immunity (SI = $10^{4.0}$) Using another syngeneic SV40-transformed cell line, ME1 C9 for immunization against ME1 C9-V15 cells

TABLE 2 *Immune State of Animals Simultaneously Exposed to Diffusion Chambers Containing 10^6 MEI C9-V15 Cells as Determined by Graded Challenge with MEI C9-V15 Cells 3 or 10 Weeks after Immunization*

Immunization				Challenge[a])						
Irrad. of cells)	Diffusion chamber		Time post immunization (weeks)	Immunized animals			Non-immunized animals			$^{10}\log$ SI[4])
	Pore size µm	Duration) (days)		Dose range	No. of animals	$^{10}\log$ TPD_{50}	Dose range	No. of animals	$^{10}\log$ TPD_{50}	
+	0.45	1	3	10^{3}–10^{7}	27	3.9	10^{1}–10^{4}	25	2.3[a]	1.6
+	0.45	3	3	10^{3}–10	27	4 2			2.3[a]	1.9
+	0 45	5	3	10^{2}–10^{6}	20	3 1	10 –10^{3}	18	2.2[b]	0.9
+	0.45	7	3	10^{3}–10^{6}	17	4.8	10^{1}–10^{4}	20	2.1	2.7
+	0 45	22–28	3	10^{2}–10^{7}	19	5.2	10^{2}–10^{4}	20	2.7[d]	2.5
—[4])	0.45	22–28	3	10^{3}–10^{7}	18	5.3			2.7[d]	2.6
+	0.45	7	10	10^{2}–10^{6}	28	3.1			2.0[e]	1 1
—[4])	0.45	7	10	10^{3}–10^{6}	27	4 1			2.0[e]	2.1
+	0 1	5	3	10^{1}–10^{4}	20	2 1			2.2[b]	−0.1
+	0.1	7	3	10^{3}–10^{6}	16	3.3			2.1	1.2
+ ; spleen cells[7])	0 1	5	3	10^{3}–10^{6}	20	3.5			2.2[b]	1.3
empty chamber[8])	0.45	22–28	3	10^{2}–10^{5}	14	2.0			2.7[d]	−0.7
+	directly inoculated		3	10^{4}–10^{6}	18	4 9			2.1	2.8
+	directly inoculated		3	10^{4}–10	20	5.7			2.7[d]	3.0
+	directly inoculated		10	10^{3}–10^{6}	15	5.1			2.0[e]	3 1
—)	directly inoculated		10	10^{2}–10^{6}	16	5.0			2.0[e]	3.0

a, b, c, d) Identical control common to several contemporary immunization groups.
) Identical pooled control from non-contemporary experiments.

as challenge, a low immunological reactivity was found on intraperitoneal (SI = $10^{0.}$) as opposed to that on subcutaneous inoculation (SI = $10^{2.3}$)

Similar experiments were also performed with xenogeneic SV40-transformed cells, R92. Doses of 10^6 cells were enclosed in diffusion

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) + = X-irradiation (10,000 R) immediately before cell inoculation or before deposition of the cells in diffusion chambers. — = non-irradiated cells were used.

) Duration of chamber deposition subcutaneously

) The animals were X-irradiated (300 R) the day before challenge.

) Sensitive index.

[a]) Leakage controls Cellular growth *in vitro* from chamber "bathings" before deposition *in vivo* = 0/20 Incidence of tumour growth at the sites of chamber deposition = 3/4 in animals challenged with 10^7 cells, 2/3 in animals challenged with 10^6 cells and 0/10 in animals challenged with 10^5–10^2 cells.

) Leakage controls: Cellular growth *in vitro* from chamber "bathings" before and after chamber passage *in vivo* = 3/29 and 4/14 respectively Incidence of tumour growth at the sites of chamber implantation (all tumours appearing before challenge) = 4 regressive and 4 progressively growing tumours out of 28 animals. Ten weeks after cell inoculation, 3 animals had a mean tumour diameter of 13 mm. By comparison, 7/19 animals directly inoculated with 10^2 cells had mean tumour diameter of 13 mm 9 weeks after cell inoculation. Less than 100 cells therefore probably leaked from the 10^6 cells enclosed in chambers.

[f]) 2 × 10^6 freshly prepared nucleated spleen cells from normal animals were added to the tumour cells in each diffusion chamber

) Chambers containing Eagle medium without cells.

[a]) All 21 animals were operated on 7 days after cell inoculation and tumours of mm-size were resected in 18 animals. In 7 animals, a second resection was performed 2 weeks after cell inoculation. Four animals died from tumours before challenge. At the time of challenge, 10 weeks after cell inoculation, the tumour incidence was 7/17 and the mean tumour diameter in tumour bearing animals was 7 mm. After challenge only the tw animals with the largest primary tumours died within 10 weeks of observation. The other tumours regressed or were stationary (about 4 mm in diameter)

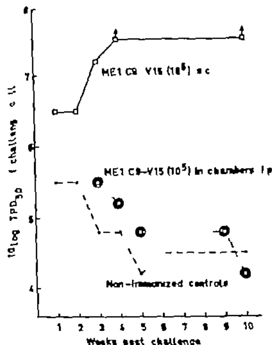


Fig 1 ^{10}log TPD_{50} of challenge cells (ME1 C9-V15) calculated weekly post challenge. The animals were inoculated 3 weeks before challenge with 10^6 irradiated ME1 C9-V15 cells subcutaneously (-□-) or were exposed for the same dose in 0 45 µm diffusion chambers deposited intraperitoneally (●) A control group was not immunized (— —) All animals were irradiated with 300 R the day before challenge.

chambers with a pore size of 0.45 µm which were deposited subcutaneously in 4-week-old mice. Another group of mice received the same cell dose subcutaneously by direct inoculation and a third group was left untreated. Three weeks later the animals were challenged with 10^4–10^6 highly oncogenic ME1 V46 cells. All non-immunized animals, 9 out of 15 animals immunized via millipore filters and 5 out of 15 directly immunized animals, developed challenge tumours (Figure 2) The difference in immune state between animals immunized directly (TPD_{50} = $10^{6.3}$ cells) and those immunized through the millipore membranes (TPD_{50} = $10^{4.7}$ cells) was not significant in contrast to the difference between each immunized group and the control ($SI \geq 10^{1.4}$)

Three-week-old animals were directly ino-

TABLE 3 *Tumour Inducing and Immunity Inducing Capacities of ME1 C9-V15 Cells Directly Inoculated Subcutaneously or Deposited Subcutaneously in Diffusion Chambers*

Immunization[1])				Challenge[2])				^{10}log Leakage quotient calculated on	
Dose range	Mode	No. of animals	^{10}log TPD_{50} (at indicated week post immunization)	Time post immunization (weeks)	Dose	No. of animals	^{10}log ImD_{50}	TPD_{50}[4]	ImD_{50}[5]
$10-10^6$	directly s.c.[3])	68	3.5 (10 w)	10	10^6	30	1.8	–2.6	–3.5
10^4-10^6	0.45 µm chambers, 7 days s.c.[4])	13	6.1 (10 w)	10	10^6	11	5.3		
10^1-10^6	directly s.c.	123	4.2 (3 w) 4.0 (9 w)	3	10^4	40	3.2	–2.3	–2.3
10^2-10^4	0.1 µm chambers, 3 days s.c.[5])	25	≥6.5 (3 w) 6.3 (9 w)	3	10^4	25	5.3		

[1]) Non-irradiated ME1 C9-V15 cells.

[2]) X-irradiation (900 R) of the animals the day before challenge.

[3]) s.c. = subcutaneously

[4]) Leakage controls Cellular growth *in vitro* from chamber bathing media before and after chamber passage *in vivo* = 2/13 (10^6 and 10^4 cells enclosed) and 1/13 (10^4 cells enclosed) respectively Incidence of tumour growth at the sites of chamber implantation = 4 regressive tumours and 3 progressively growing tumours in 13 animals (10^4–10^6 cells enclosed)

[5]) Leakage controls Cellular growth *in vitro* from chamber bathing media before and after chamber passage *in vivo* = 0/25 and 2/25 (10^4 cells enclosed) respectively Tumour incidence at the sites of chamber implantation = 1/25 (10^4 cells enclosed)

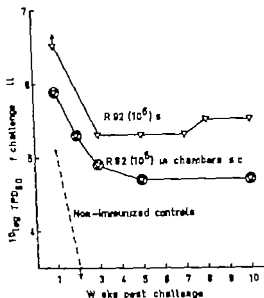


Fig 2 ^{10}log TPD$_{50}$ of challenge cells (ME1 V46) calculated weekly post challenge. Three weeks before challenge the animals were inoculated with 10^6 R92 cells subcutaneously (—▽—) or were exposed to 10^6 R92 cells in 0 45 μm diffusion chambers deposited for 2-4 weeks subcutaneously (—⊙—) A control group was not immunized (— — —)

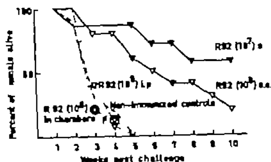


Fig 3 Percent of animals surviving the challenge of 10^7 ME1 V46 cells, calculated weekly post challenge. Three weeks before challenge, the animals were inoculated with 10^7 R92 cells subcutaneously (—▼—) 10^6 R92 cells subcutaneously (—▽—) 10^6 R92 cells intraperitoneally (▽) or were exposed to 10^6 R92 cells enclosed in 0.45 μm diffusion chambers deposited intraperitoneally (⊙) A control group was not immunized (— — —)

culated intraperitoneally with 10^6 R92 cells or had a millipore diffusion chamber containing the same number of cells deposited intraperitoneally Positive controls were immunized with 10^7 or 10^6 R92 cells subcu taneously and negative controls were not immunized at all. Three weeks after immunization, all animals were challenged with 10^7 highly oncogenic ME1 V46 cells. Eight teen animals immunized intraperitoneally and 10 non-immunized animals were killed by their challenge tumours at the same rate and all of them died within 5 weeks (Figure 3) The tumours in animals immunized subcutaneously grew more slowly and 6 out of 17 animals were still alive 10 weeks after challenge. The difference in times of death of animals immunized with 10^6 or 10^7 cells subcutaneously and the other groups combined was statistically significant ($p<0.05$ Wilcoxon unpaired test)

In a separate experiment the SV40-specificity of the R92 cells was tested. 10 non-transformed spleen cells from the same strain of rats (Sprague-Dawley) as the R92 cells did not immunize when inoculated subcutaneously (SI = $10^{0.2}$) whereas 10^7 R92 cells did, as expected (SI$\geq 10^{1.4}$)

DISCUSSION

Diffusion chambers have been found to be a useful tool in studies of cellular and humoral pathways for mediation of immunity against transplantation antigens (*Prehn et al* 1954, *Algire et al.* 1954 *Ambrose et al.* 1971) They enable the contact with the source of antigen to be interrupted at a desired point and the porosity can be chosen so as to prevent or allow the penetration of im mmocompetent cells. A disadvantage is that a leakage of cells might occur and must be carefully checked.

In the present study in which a threshold dose (10^6) of irradiated syngeneic SV40-transformed cells (ME1 C9-V15) was enclosed in 0 45 μm diffusion chambers and deposited subcutaneously the implantation time necessary to induce immunity was determined by challenge 3 weeks later For induction of a high level of immunity equal to that produced by the same number of cells inoculated directly no more than 7 days of implantation

was required, and only one day was sufficient for demonstrating a significant immunity This was consistent with the fact that animals immunized subcutaneously showed full strength immunity when challenged as soon as after 7 days. In a tumour system studied by *Snell et al.* (1960) the minimum sensitization time for tumour immunity to be passively transferable by lymphoid cells was 4 days and *Dvorak & Waksman* (1962) reported that lymphoid cells in diffusion chambers became slightly sensitized to transplantation antigens after 4 days and strongly sensitized thereafter

Leakage of cells might play a role in the development of immunity (*Algire & Moore* 1959) To minimize this risk, a treshold dose (10^5) of irradiated ME1 C9-V15 cells was used. It has been shown (*Stillström* 1973 and 1974) that halving of the subcutaneous inoculation dose ($10^{4.7}$ cells) decreased the immunization effect one hundred times ($SI = 10^{0.9}$) and a ten-fold reduction (to 10^4 cells) decreased it more than one thousand times. The leakage of cells was evaluated by cultivation of culture medium bathing chambers containing non irradiated cells, before and after passage *in vivo* and by calculation of leakage quotients.

The small numbers of tumour cells found by growing the bathing media indicated that the rate of leakage was low A low rate ("a few cells") and a high incidence of leakage (about 30 per cent of the chambers) were found by *Algire & Moore* (1959) who used highly oncogenic leukaemic cells in 0 45 μm diffusion chambers. In the present study the leakage quotient based on TPD_{50} of directly inoculated and chamber enclosed cells (7 days in 0 45 μm chambers deposited subcutaneously) was too low ($10^{-4.}$) for leakage to have caused the immunization effect of the irradiated ME1 C9-V15 cells deposited in chambers for 7 days. Some other factor of immunization must have been involved e.g subcellular transplantation antigens leaving the chambers or immunocompetent cells invading them.

The high level of immunity found in animals bearing a diffusion chamber for at least one week and challenged 3 weeks after chamber insertion contrasted to the low level of immunity in animals challenged 10 weeks after immunization with cells contained in diffusion chambers. The decrease in immunity was possibly caused by the removal of the source of antigen or by the removal of significant numbers of sensitized lymphoid cells or memory cells along with the chambers. After immunization with the same number of irradiated cells inoculated subcutaneously no decrease in the level of immunity was recorded after 10 weeks. The low ability of chamber enclosed cells to induce a long-lasting immunity was also shown by the high ImD_{50} ($10^{5.3}$ cells) determined by challenge 10 weeks after a 7-day chamber deposition. Directly inoculated cells were more than one thousand times ($10^{3.6}$ times) more effective ($ImD_{50} = 10^{1.8}$ cells)

There was an increase in the level of immunity with duration of chamber deposition both when chambers with 0.45 μm pores and chambers with 0 1 μm pores were used. The SI rose from $10^{0.8-1.9}$ after deposition periods of less than 7 days to $10^{2.0-2.7}$ after 7 days or more when 0 45 μm membranes were used and from $10^{-0.1}$ to $10^{1.3}$ when 0.1 μm membranes were used. It is probable that this change reflects an immunological process depending on an accumulation of cell-dissociated transplantation antigens *outside* the chambers or depending on the maturation of a cellular immune response at the site of implantation.

According to *Capalbo et al.* (1964) millipore chambers with 0 45 μm pores are not completely cell impermeable about 70 per cent of the chambers in their study were found to contain an average of 4,200 host cells after 2–16 days intraperitoneally Chambers with 0 1 μm pores, on the other hand, were not penetrated. A similar result was achieved by *Stutman et al.* (1969) *Dvorak & Waksman* (1962) were able to sensitize normal lymphoid cells against transplantation antigens inside diffusion chambers. The fact that normal spleen cells inside diffusion chambers

with 0.1 µm pores containing irradiated ME1 O9-V15 cells induced immunity which was not possible with antigenic cells alone, showed that lymphoid cells in contact with the tumour cells within the chambers might be responsible for immunization effects even without leaving the chambers. Some of the immunization effects with cells in 0.45 µm chambers could have been due to the penetration of lymphoid cells, but this was less probable for 0.1 µm chambers.

In the present work, intraperitoneal immunization with SV40-transformed cells either contained in 0.45 µm diffusion chambers or inoculated directly was not effective. This was in agreement with the results obtained by *Prehn et al.* (1954) who found no immunization effect of antigenic tumour cells in 1 µm chambers deposited intraperitoneally and with those obtained by *Biggs & Eiselein* (1965) who were not able to immunize with Ehrlich ascites cells in 0.45 µm chambers placed intraperitoneally unless the cells were first virus infected. *Dubreuil et al.* (1972) who immunized hamsters with SV40 transformed cells, showed that subcutaneous immunization was more effective than intraperitoneal. *Bainbridge & Gowland* (1971) investigated the intraperitoneal route of immunization against allogeneic transplantation antigens and found that blockade of the reticulo-endothelial system of the liver resulted in an increased immunization effect. It was assumed that the reticulo-endothelial system interfered with immunization by removing antigenic material from the cells.

Thus the results obtained in the present and other studies show that the subcutaneous route is much more suitable for immunization against transplantation antigens than the intraperitoneal route as regards both directly inoculated cells and cells enclosed in diffusion chambers.

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DECREASED ONCOGENIC POTENTIAL OF SV40-TRANSFORMED CELLS GROWN IN DIFFUSION CHAMBERS INTRAPERITONEALLY

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Small doses (10^4) of cells originating from two cloned lines of SV40-transformed mouse embryo cells, ME1 C9 and ME2 C11 were enclosed in diffusion chambers which were deposited intraperitoneally in syngeneic CBA mice treated in different ways. The cells derived from the chambers after 3–84 days *in vivo* were less oncogenic than the cells of origin in 26 cases out of 29 The decrease in oncogenic potential was most pronounced (Progression index, PI = $10^{-1.4}$–$10^{-8.7}$) in irradiated animals and least pronounced (PI = $10^{-0.3}$–$10^{-1.4}$) in non-irradiated tumour-bearing animals. Cells passaged subcutaneously and harvested from the resulting tumours after 7–27 weeks were more oncogenic than the cells of origin in 20 cases out of 23 A decrease in oncogenic potential was only found in lines derived from tumours induced by a small dose (10^3–10^4 cells) in irradiated animals (*PI* = $10^{-2.4}$ $\leq 10^{-4.6}$) *Lines derived from tumours induced by a large dose* (10^5 *cells*) in irradiated animals or small dose (10^2–10^3 cells) in non-irradiated animals were more oncogenic than the cells of origin (PI = 10^0–$10^{1.8}$)

It is well-known that rat and mouse cells may become oncogenic spontaneously or after virus transformation, after many months of *in vitro* propagation (*Gey* 1941 *Earle* 1943) and that *in vivo* passage of cells forming tumours increases the oncogenic potential of the cells (*Sanford et al.* 1954) An increased oncogenic potential after subcutaneous passage was found by *Enders & Diamandopoulos* (1969) in 5 cloned lines of SV40-transformed hamster cells. The opposite event, a decrease of the oncogenic potential, has sometimes happened *in vitro* (*DeBruyn & Gey* 1952 *Eng et al.* 1971 *Ting et al.* 1972) and methods have been developed for an intentional selection *in vitro* of cells with a reduced oncogenic potential (*Pollack et al.* 1968 *Rabinowitz & Sachs* 1968)

The aim of the present investigation was to distinguish the conditions that might regulate the oncogenic potential of SV40-transformed cells of syngeneic origin *in vivo* To allow recovery of cells with all degrees of malignancy in a certain time, even those with a weak oncogenic potential, cells were enclosed in millipore diffusion chambers which were deposited intraperitoneally Different factors which might possibly influence the tumour progression were studied, i.e. the number the oncogenic potential and the antigenicity of the cells and environmental factors such as irradiation or immunization of

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the recipient animals or growth of an SV40 tumour elsewhere in the body

MATERIAL AND METHODS

Virus

A strain of SV40, obtained from Dr Magrath Research Council Laboratories, London was used for transformation of cells.

Animals

CBA mice from the Department of Genetics, University of Stockholm, were used. Cell cultures were prepared from whole embryos. The oncogenic potentials of different cell lines were determined in 5-week-old irradiated male mice.

Cells

Two stem lines of CBA mouse embryo cells were employed. The first, ME1 originated from SV40-transformed cells from one embryo. It was non-oncogenic for many months, but finally produced tumours when large numbers of cells were inoculated into irradiated syngeneic animals (*Wesslén* 1970) After about 2 years and 300 passages *in vitro* the ME1 line was still only weakly oncogenic. It was then cloned and one of the clones, ME1 C9 and its 15th *in vivo* tumour passage cell line, ME1 C9-V15 were used in the present and preceding investigations (*Wesslén* 1970 *Stillström* 1973) The second stem line ME2, originating from tissues of 15-day-old embryos, was transformed by SV40 in the second *in vitro* passage. A large colony with a transformed appearance was harvested 5 weeks after SV40 infection of the cells and passaged for 7 months until the cells were cloned (ME2 C11) The ME1 ME1 C9 ME1 C9-V15 and ME2 C11 cells contained T-antigen in all cell nuclei, according to immunofluorescent tests with FITC-cojugated hamster anti-SV40-T-globulin (Flow Laboratories, Irvine Scotland)

Cloning

The mehtod of *Martin & Tuan* (1966) was followed. Cells were seeded in plastic petri dishes with small square coverslips (5 × 5 mm) After adherence of cells, a coverslip with only a few cells attached was transferred to a new petri dish containing culture medium. All cells except one were scraped from the glass by hand, using sterile disposable needles. The glass was held with tweezers and the manipulation was controlled in an inverted microscope. The glass was then again transferred to a new dish containing fresh growth medium. The growth of the clone was inspected daily and cells that seemed to creep up from the edges of the glass or that appeared distant from the clone were scraped off

Passages in vitro

The cells were subpassaged 3 times in two weeks. When stabilized, all lines could be split at least 1:100 at each passage. The culture medium was Eagle's medium supplemented with 10 per cent calf serum. The cells were grown in stationary glass bottles or plastic petri dishes at a temperature of 36.5 C in an atmosphere of about 5 per cent CO_2 in air 0.25 per cent trypsin in PBS was used to loosen the cells.

Passages in Millipore Diffusion Chambers Intraperitoneally

Nylon reinforced millipore filter (TWWP Millipore, Bedford, Mass., U.S.A.) with a thickness of 100 µm and a pore size of 0.45 µm, and plexiglass rings (2 mm high and with an external diameter of 14 mm) were used to construct diffusion chambers. Filters and half-completed chambers with one filter disc glued to the ring were kept at 60 C for 1–2 days. 10^4 cells in a volume of 0.05 ml were gently placed in the middle of the basin formed by the ring and the "bottom filter and a second, "top" filter was cemented to the ring the chamber was thus complete. The animals were anaesthetized with ether the abdominal skin was shaved and cleaned with alcohol and a lateral incision was made through the skin and peritoneum. The diffusion chamber was placed in the peritoneal cavity and the wound was closed with silk sutures. The chambers were left in situ for up to 23 days, in most cases for 14 days, and caused no apparent discomfort. They often became adherent to abdominal organs. The chambers were removed aseptically under ether anaesthesia. After removal they were first bathed in Eagle's medium for a few minutes and were then immersed in a 0.25 per cent trypsin solution for 5 minutes. Before tearing off one of the filters, the exterior of the chamber was cleaned with cotton-woll swabs soaked in 0.25 per cent trypsin solution. The interior of the chamber was often filled with a yellow clot. Available cells were transferred to a petri dish with medium. After at least 4 passages *in vitro* the derived cells were tested for their oncogenic potential or re-passed in millipore chambers. Between every two consecutive millipore chamber passages the cells were cultivated in 4–11 passages in vitro.

Passages S bcutaneously

Cells inoculated into animals were always grown in tissue culture and were never passed directly from one animal to another They were harvested by gentle trypsinization, suspended in Eagle's medium, washed by a single centrifugation (670 × g) and resuspended in Eagle's medium, counted in a haemocytometer and dil ted to an appropriate cell concentration. The cells were inoculated subcu-

taneously in a volume of 0.5 ml. Tumours, which always appeared at the site of injection, were inspected every 7 days after cell inoculation. The tumour diameter was recorded as the mean of the longest axis and the largest breadth at right angles to it. Tumours were harvested after different periods of growth. They were excised aseptically and pieces of firm, non-necrotic tumour were minced in a small volume of Eagle medium. The mince was flushed several times through a syringe and seeded in petri dishes. The medium was changed the day after explantation and every second day thereafter until the tumour line was established.

Oncogenic Potential

The oncogenic potential of a cell line, expressed as the 50 per cent tumour producing dose, TPD_{50}, was determined by the subcutaneous inoculation of graded doses of cells (in 10-fold dilution steps) in 3-week-old male animals which had been X-irradiated the day before cell inoculation. The X-ray dose was 300 R if not otherwise specified in "Results". There were generally 5 animals per dose group and 4–7 dose groups per titration. The animals were examined weekly for tumours. Tumours 4 mm in diameter or larger were included in the TPD_{50} calculations which were performed according to *Kärber* (1931). The tumour progression or the increase in the oncogenic potential of the cells was expressed as the progression index, PI (*Ställström* 1973), i.e. the ratio of the TPD_{50} of the original line to that of the derived line. An index below 1 meant a decreased oncogenic potential of the derived cells compared with the cells of origin. A PI≥10 or≤0.1 was considered to indicate a significant change of the oncogenic potential (*Ställström* 1973).

Immunogenicity

In tests of immunogenicity animals inoculated with 10^6 or 10^7 irradiated cells were challenged 3 weeks after immunization together with non-immunized controls, and the sensitivity index, SI (*Ställström* 1973) was determined. The SI is the ratio of the TPD_{50} of challenge cells in immunized to that in non-immunized animals. A non-significant immunization effect (SI<10) may only indicate that a threshold dose of immunization has not been reached (*Ställström* 1974). ME1 C9-V15 was the cell line most often used for challenge (cf. Results). By the 50 per cent immunizing dose, ImD_{50}, is meant the dose of immunizing cells which is required to make 50 per cent of the animals resistant to defined challenge, usually 10 ME1 C9-V15 cells (*Ställström* 1974).

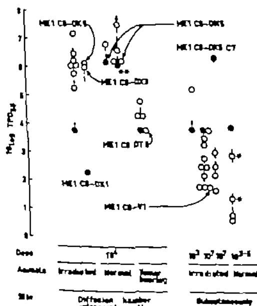


Fig. 1. Change (● – – ○) in oncogenic potential (^{10}log TPD_{50}) of ME1 C9 and ME1 C9 derived cells (● type of line stated if not ME1 C9) in different types of passages *in vivo*. Pore size of diffusion chambers = 0.45 μm. Tumours were produced by ME1 C9 cells. Animals (those indicated) were X-irradiated with 300 R the day before chamber implantation or cell inoculation. Oncogenic potentials of original (●) and derived (○) lines were determined subcutaneously in 3-week-old animals irradiated with 300 R the day before cell inoculation (exceptions: * = non-irradiated, ** = irradiated with 200 R the day before titration).

RESULTS

Oncogenic Potential after Passages in Millipore Diffusion Chambers Intraperitoneally

In irradiated animals. 10^4 ME1 C9 cells were enclosed in millipore chambers in animals irradiated with 300 R. All lines derived from single 14-day maintenance in intraperitoneal diffusion chambers were tested before their 14th *in vitro* passage and found to be much less oncogenic than the original ME1 C9 cells (Figure 1). The TPD_{50}s of 7 lines varied between $10^{4.3}$ and $10^{7.3}$ cells, whereas that of the ME1 C9 stem line carried *in vitro* changed from $10^{3.8}$ to $10^{2.3}$ cells during the experimental period.

In one experiment, the ME1 C9 cells were enclosed for 13 days in a chamber and de-

the recipient animals or growth of an SV40 tumour elsewhere in the body

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The cells were subpassaged 3 times in two weeks. When stabilized, all lines could be split at least 1 100 at each passage. The culture medium was Eagle's medium supplemented with 10 per cent calf serum. The cells were grown in stationary glass bottles or plastic petri dishes at a temperature of 36.5 C in an atmosphere of about 5 per cent CO_2 in air. 0.25 per cent trypsin in PBS was used to loosen the cells.

Passages in Millipore Diffusion Chambers Intraperitoneally

Nylon-reinforced millipore filter (TWWP Millipore, Bedford, Mass., U.S.A.) with a thickness of 100 μm and a pore size of 0.45 μm, and plexiglass rings (2 mm high and with an external diameter of 14 mm) were used to construct diffusion chambers. Filters and half-completed chambers with one filter disc glued to the ring were kept at 60 C for 1–2 days. 10^4 cells in a volume of 0.05 ml were gently placed in the middle of the basin formed by the ring and the "bottom" filter, and a second, "top" filter was cemented to the ring; the chamber was thus complete. The animals were anaesthetized with ether; the abdominal skin was shaved and cleaned with alcohol and a lateral incision was made through the skin and peritoneum. The diffusion chamber was placed in the peritoneal cavity and the wound was closed with silk sutures. The chambers were left in situ for up to 23 days, in most cases for 14 days, and caused no apparent discomfort. They often became adherent to abdominal organs. The chambers were removed aseptically under ether anaesthesia. After removal they were first bathed in Eagle's medium for a few minutes and were then immersed in a 0.25 per cent trypsin solution for 5 minutes. Before tearing off one of the filters, the exterior of the chamber was cleaned with cotton-wool swabs soaked in 0.25 per cent trypsin solution. The interior of the chamber was often filled with a yellow clot. Available cells were transferred to a petri dish with medium. After at least 4 passages *in vitro*, the derived cells were tested for their oncogenic potential or re-passed in millipore chambers. Between every two consecutive millipore chamber passages the cells were cultivated in 4–11 passages in vitro.

Passages Subcutaneously

Cells inoculated into animals were always grown in tissue culture and were never passed directly from one animal to another. They were harvested by gentle trypsinization, suspended in Eagle's medium, washed by a single centrifugation (670 × *g*) and resuspended in Eagle medium, counted in a haemocytometer and diluted to an appropriate cell concentration. The cells were inoculated subcu-

ME2 C11 cells. These two animals were considered immune because the 50 per cent immunizing dose, ImD_{50}, of these cells against 10^6 ME1 C9-V15 cells was $10^{3.3}$ cells. The $TPD_{50}s$ of the derived lines were $10^{6.3}$ and $10^{6.1}$ cells (Figure 2) thus not significantly different from that of the original line.

Oncogenic Potential after Passages Subcutaneously

Cells from different lines were inoculated subcutaneously. The resulting tumours were harvested after different times and the explanted tumour cells were allowed to initiate cell lines *in vitro*. The oncogenic potential of a derived line was determined after a few passages *in vitro*.

Seventeen lines derived from tumours induced by 10^7 cells in irradiated animals had $TPD_{50}s$ of $10^{1.4}$–$10^{4.8}$ cells. They were significantly ($PI \geq 10$) more oncogenic than their lines of origin in 13 cases and more or equally oncogenic ($0 \leq PI < 10$) in the remaining 4 cases (Figures 1 and 2).

Two lines derived from tumours induced by 10 and 10^4 cells in irradiated animals had $TPD_{50}s$ of $10^{6.3}$ and $\geq 10^{6.5}$ cells. The lines were less oncogenic than their lines of origin, with PIs of $10^{-1.4}$ and $\leq 10^{-6.6}$ (Figures 1 and 2).

Four lines derived from tumours induced by 10^5 to 10^6 ME1 C9 cells in non-irradiated animals had $TPD_{50}s$ of $10^{3.7}$–$10^{2.3}$ cells in non-irradiated animals, which meant a tumour progression of $10^{1.8}$–$10^{3.3}$ in comparison with the oncogenic potential ($TPD_{50} = 10^{5.5}$) of the original cells, measured in non-irradiated animals (Figure 1).

Oncogenic Potential after Passages in Vitro

The cloned cell line ME1 C9 was more oncogenic than the ME1 cells from which it was derived and its oncogenic potential increased further with time, from a TPD_{50} of $10^{7.4}$ cells or more to $10^{4.9}$ cells in non-irradiated animals and from $10^{3.3}$ to $10^{3.3}$ cells in irradiated animals, in 3 years and more than 300 passages *in vitro*. Re-cloning of the cells after more than 300 passages showed that the ME1 C9 cells were heterogeneous as regards oncogenic potential. In clone was more than 100 times as oncogenic as another clone ($TPD_{50}s \leq 10^{1.3}$ and $10^{3.9}$ cells, respectively).

Seven lines derived from the ME2 cells did not produce progressively growing tumours when tested 2–45 passages after establishment. One of the lines, the clone ME2 C11, was not oncogenic after 65 passages but was found to be weakly oncogenic ($TPD_{50} = 10^{6.1}$ cells) in irradiated animals after 128 passages.

Some of the lines derived in this study were titrated for oncogenic potential several times. The ME1 C9-DX1 line became highly oncogenic ($TPD_{50} = 10^{1.3}$) before 47 passages *in vitro*. The ME1 C9-DN5 line was weakly oncogenic after 73 passages ($TPD_{50} = 10^{6.3}$) but highly oncogenic ($TPD_{50} = 10^{1.3}$) after 132 passages. The ME1 C9-DX5 line was weakly oncogenic after 10, 30 and 110 passages ($TPD_{50}s = 10^{6.1}$, $10^{6.6}$, $10^{6.6}$ cells). The line was cloned after 62 passages and the clone, ME1 C9-DX5 C7 had the same low oncogenic potential ($TPD_{50} = 10^{6.3}$) as the line of origin.

Immunogenicity of Derived Cell Lines

The ME1 C9-DX5 C7, which was a weakly oncogenic clone of the weakly oncogenic 5th millipore chamber passage of ME1 C9 cells in irradiated animals, the ME1 C9-DN5 line, an initially weakly oncogenic line of ME1 C9 cells passed 5 times intraperitoneally in millipore chambers in non-irradiated animals and the ME1 C9-V1 line, a highly oncogenic first tumour passage of ME1 C9 (Figure 1) were all weakly immunogenic against a challenge of ME1 C9-V15 cells. The SIs of the challenge cells varied between $10^{0.9}$ and $10^{1.4}$ (Table 1). The immunogenicity of the ME2 C11 line was equally weak ($SI = 10^{0.8-1.1}$) when 10^8 and 10 irradiated cells were used for immunization (Table 1). After one tumour passage of the ME2 C11 cells, the derived tumour line, ME2 C11 V1 was more than 100 times as oncogenic as the

TABLE 1 *Immunogenicity of ME1 C9 Derived Cell Lines ME2 C11 ME2 C11 V1 and ME3 C1C1 Cell Lines*

Immunization			Challenge							
				Immunized animals			Non-immunized animals			
Cell line	Dose[1])	OP[2])	Cell line	Dose range	No. of animals	^{10}log TPD$_{50}$[3])	Dose range	No. of animals	^{10}log TPD$_{50}$	^{10}log SI[4])
ME1 C9-DN5 C7[a])	10^6	6.3	ME1 C9-V15	10^1–10^7	33	3.3	10^1–10^4	20	2.5	0.8
ME1 C9-DN5[a])	10^6	6.2	ME1 C9-V15	10^4–10	30	4.1	10–10^5	25	2.7	1.4
ME1 C9-V1)	10^6	1.8	ME1 C9-V15	10^3–10^7	30	4.1	10^1–10^5	25	2.7)	1.4
ME2 C11)	10^7	5.4	ME1 C9-V15	10–10^6	26	2.9	10^1–10^4	19	1.9b)	1.0
ME2 C11	10^6		ME1 C9-V15	10^4–10^7	29	3.8			2.7)	1.1
ME2 C11	10^6		ME1 C9-V15	10^6–10^7	22	3.6	10^1–10^7	35	2.7c)	0.9
ME2 C11 V1)	10^6	2.7	ME1 C9-V15	10^3–10^7	25	3.5			2.7)	0.8
ME3 C1C1[7])	10	1.5	ME1 C9-V15	10^1–10^6	23	2.3			1.9b)	0.4
ME2 C11	10^7		ME2 C11 V1	10^3–10^7	25	4.4	10^2–10^7	30	4.4d)	0.0
ME2 C11 V1	10^6		ME2 C11 V1	10^4–10^7	23	4.0			4.4d)	−0.4

) Number of irradiated (10,000 R) cells inoculated subcutaneously 3 weeks before challenge.
[2]) OP = Oncogenic potential of the cell line, expressed as the TPD$_{50}$ (log units) in 3-week-old irradiated (300 R) mice.
[3]) TPD$_{50}$ = Number of cells necessary for 50 per cent tumour take, from readings 10 weeks post challenge.
[4]) Sensitivity index = Ratio of the TPD$_{50}$ of challenge cells in immunized animals to the TPD$_{50}$ of the same cells in non-immunized (control) animals.
) Cf. Figure 1
) Cf. Figure 2.
[7]) SV40-non-transformed mouse embryo cells, cloned twice.
b d) Identical control groups.

cell line of origin (Figure 2) but the immunogenicity of the derived line was the same ($SI = 10^{0.3}$ Table 1) No immunization was induced by 10^{6} irradiated ME2 C11 or ME2 C11 V1 cells against a challenge of ME2 C11 V1 cells ($SI = 10^{0.6}$ and $10^{-0.4}$ respectively)

DISCUSSION

The confinement of cells within a millipore chamber deposited intraperitoneally efficiently precluded tumour progression of all the 29 lines derived from chambers in animals treated in different ways, none was significantly more oncogenic ($PI_{max} = 10^{0.3}$) than the original cells. On the contrary many lines were significantly less oncogenic. These observations were in accordance with those of *Parmiani et al.* (1971) who found that primary cultures of mouse tissues did not acquire an oncogenic potential in millipore chambers *in vivo* for up to 46 weeks. Cells cultivated in millipore chambers *in vitro* however became more oncogenic after 16 weeks. The authors therefore concluded that *in vivo* fluids must contain some factor able to delay neoplastic tranformation. It is also possible that the chambers, by setting bounds to the size of the cell population, reduce the number of appearing highly oncogenic mutant cells significantly and that the growth conditions in the chambers are more favourable to weakly oncogenic cells than to highly oncogenic cells. Even without confinement of the cells in chambers, small cell populations (10^{3} and 10^{4} inoculated cells) became less oncogenic in two irradiated animals and thus, the chambers may function only as a device which facilitates the recovery of cells with a low oncogenic potential.

After a single chamber passage in irradiated, non-immunized animals, moderately or highly oncogenic ($TPD_{50} = 10^{3.6}$–$10^{5.0}$ cells) ME1 C9 cells became significantly ($PI \leq 0.1$) less oncogenic in 8 cases out of 8. The TPD_{50} varied between $10^{5.3}$ and $10^{7.3}$ cells and the PI between $10^{-1.6}$ and $10^{-3.7}$ Additional passages in millipore chambers deposited intraperitoneally after the cells had achieved a low oncogenic potential did not influence the oncogenic potential any further (Figure 1) Probably the oncogenic potential could not be completely lost.

Definite progression indices could not be calculated for the ME1 C9 cells used for millipore chamber passage in non-irradiated non-immunized animals, because the oncogenicity titrations of the original cells were made in animals irradiated with 200 R instead of 900 R and because one line was too weakly oncogenic to give a definite TPD_{50} value ($\geq 10^{7.4}$ cells, Figure 1) In comparison with ME1 C9 cells kept *in vitro* the cells derived from the millipore chambers were, however significantly less oncogenic in 3 out of 4 cases.

ME1 C9 derived lines from 4 millipore chambers deposited in animals bearing ME1 C9 tumours had TPD_{50} between $10^{4.4}$ and $10^{6.9}$ cells. Only one line was significantly less oncogenic than the cells of origin. ME2 C11 cells passaged in millipore chambers in animals bearing ME2 C11 tumours or immunized with ME2 C11 cells had TPD_{50} between $10^{5.3}$ and $10^{6.2}$ cells in 6 lines. No line was significantly less oncogenic than the cells of origin (Figure 2)

The rate of successful recovery of cells from diffusion chambers was lower in chambers from irradiated animals than in those from non irradiated animals. This may reflect a more efficient selection of cells in irradiated animals or the fact that the cells were normalized and therefore should have been more adherent to the supporting surface and should have grown less densely than tumour cells.

Seventeen lines from tumours induced by 10^{7} cells in irradiated animals were more or equally oncogenic in relation to the cells of origin and had TPD_{50} between $10^{1.8}$ and $10^{4.4}$ cells, whereas 2 tumour lines induced by 10^{3} and 10^{4} cells were less oncogenic than their cells of origin and had TPD_{50} above 10^{6} cells. The number of original cells inoculated therefore seemed to be of importance for the oncogenic potential of the derived

cell lines. Four cell lines from tumours induced by 10^4–10^5 cells in non irradiated animals were more oncogenic than their cells of origin with TPD_{50} below 10^3 cells in non-irradiated animals. A small inoculation dose and irradiation of the animals seemed to be factors which combined resulted in a reduced oncogenic potential of the inoculated cells.

Some of the tumour lines in the present study had reached an oncogenic potential on a level with the ME1 C9-V15 line which was derived from the 15th tumour passage of ME1 C9 cells. It was therefore evident that considerable tumour progression may take place during a single subcutaneous passage. Tumour progression was possible both in a highly immunogenic, moderately oncogenic line (ME1 C9) and in weakly immunogenic, weakly oncogenic lines (ME1 C9-DX5 C7 and ME2 C11)

The tumour progression of ME1 C9 cells *in vivo* could be due to a selection of pre-existing highly oncogenic cells *in vitro* because this line was cloned more than 200 passages before subcutaneous inoculation and the line was shown by cloning to contain cells with different oncogenic potentials. In spite of the fact that the ME1 C9 DX5 C7 line was recently cloned (passage 5) from the stably low-oncogenic ME1 C9 DX5 line (Figure 1) when used for subcutaneous passage, the 4 lines derived from the resulting tumours were more than thousand times as oncogenic as the cells of origin and the tumour progression thus even greater than that in the ME1 C9 line. If the tumour progression of the ME1 C9-DX5 C7 cells really was caused by selection of highly oncogenic cells, the variability in oncogenic potential might have arisen *de novo* after subcutaneous inoculation of the cells. This was in accordance with *Enders & Diamandopoulos* (1969) who showed that non-cloned SV40-transformed hamster cells increased their oncogenic potential after one subcutaneous tumour passage on an average $10^{1.6}$ times in 4 experiments and that all of 5 cloned lines became more oncogenic (on an average $10^{2.3}$ times) after one subcutaneous passage.

SV40-transformed cells were found on the outer surface of two millipore chambers with ME2 C11 cells after intraperitoneal passage in animals with subcutaneous tumours induced by these cells. The cells outside the chambers were more oncogenic (PI = $10^{1.3}$ and $10^{2.6}$ respectively) than the original ME2 C11 cells and the cells retained within the chambers (Figure 2) This finding may indicate that even small numbers of cells in a non-irradiated tumour bearing animal may increase their oncogenic potential in a short time before visible tumour formation. If the "leakage" cells originated from the established tumours, their TPD_{50}s would nevertheless indicate that the increase in the oncogenic potential comprised disseminated cells at some distance from the main tumour

In the present study there was a general trend towards an increased oncogenic potential in cell lines kept *in vitro* The change was slower than the tumour progression *in vivo*. This was in agreement with the results of *Enders & Diamandopoulos* (1969) who did not find an increased oncogenic potential in three cell lines after only 30 passages *in vitro*.

A partial loss of transformed cell properties, as in the present investigation, has been produced by different means in different tumour systems. *Rabinowitz & Sachs* (1968, 1970) reverted polyoma-transformed hamster cells to a lower oncogenic potential by growing them on monolayers of fixed normal cells. *Pollack et al.* (1968) selected SV40-transformed mouse embryo cells with increased contact inhibition of growth by killing non-contact inhibited cells in DNA synthesis by fluorodeoxyuridine. The selected cells were normalized in many parameters of growth and showed a reduced oncogenic potential. *Rothschild & Black* (1970 1973) cultivated cloned SV40-transformed hamster cells in the presence of 5-bromodeoxyuridine and produced new cell lines with a decreased oncogenic potential.

The ME1 C9 and ME1 C9-V15 cell lines have been shown to be highly immunogenic and immunosensitive in tumour cell transplantation experiments *(Ståhlström* 1973 and

1974) Three lines derived from passages of ME1 C9 cells in vivo were significantly less immunogenic than the ME1 C9 and the ME1 C9-V15 cells (Table 1) One line was more oncogenic and one line was less oncogenic than the cells of origin and one line was weakly oncogenic before but highly oncogenic after the time of the immunization experiment. It is possible that these derived lines had lost SV40 or other transplantation antigens. All the derived lines were however still SV40-T-antigen positive. The ME2 C11 line and its more oncogenic tumour passage line were of the same low immunogenicity as the ME1 C9 derived lines mentioned above and probably had never had that "extra" dispensable transplantation antigen which could be supposed to exist in the ME1 C9 and ME1 C9-V15 cells.

The tumour progression in the ME2 C11 system, like that in the ME1 C9 system studied earlier (*Stillström* 1973) was not associated with any change in the immunogenicity of the cells. The oncogenic potential and transplantation antigenicity may therefore vary independently

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IN VITRO INFLUENCE OF GLUCOCORTICOSTEROIDS ON OPSONIC AND BACTERICIDAL ACTIVITIES AGAINST *ESHERICHIA COLI* IN RAT SERUM

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The *in vitro* influence of methylprednisolone and hydrocortisone on rat humoral defence factors against *E. coli* was evaluated by means of radioisotope technique using ^{32}P-labelled *E. coli*. The opsonic activity of serum was studied by measuring uptake of bacteria by glass-adherent polymorphonuclear neutrophils from rats. The drug influence on bacteria and phagocytes was also evaluated. Pre-incubation of phagocytes with methylprednisolone did not reduce the cellular uptake, and pre-incubation of bacteria with this drug did not affect the rate of release of label from bacteria. Concentrations of methylprednisolone higher than 1 mg per ml blocked the opsonic and bactericidal activities of serum, and this effect was independent of pre-incubation.

The infection-enhancing effect of long term treatment with glucocorticosteroids (steroids) might be due to impairment of host defence factors or to a direct effect on microorganisms.

Impaired engulfment of inert particles has been observed at very high hydrocortisone concentrations *in vitro* (4) The influence of steroids on the ability of polymorphonuclear neutrophils (PMN) to engulf bacteria appears more uncertain (2) An impaired opsonic effect of serum due to steroids has not been clearly demonstrated. Anticomplement properties of steroids have been observed in an immune haemolytic system (3) while the union of antigen and antibody does not appear to be prevented by these drugs (4) Their influence on the complement-mediated bactericidal reactions has not been extensively investigated

The aim of the present study has been to evaluate the influence of steroids on rat humoral defence factors against *E. coli*. Using ^{32}P labelled *E. coli* according to previously described methods (1 6) the drug effect on bactericidal activity of serum, and on its ability to induce uptake of *E. coli* by PMN was evaluated *in vitro* Furthermore, the influence of methylprednisolone on PMN and bacteria was studied.

MATERIALS AND METHODS

Chemicals

Methylprednisolone succinate (Solu-Medrol®) and hydrocortisone succinate (Solu-Cortef®) (The Upjohn Company Kalamazoo, Mich., U.S.A.) were dissolved in a Krebs-Ringer phosphate buffer

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with 10 mM glucose (KRG) (6) at pH 7.2–7.4. A complex of bis-hydrocortisone (21)-sodiumphosphate-hydrocortisone ((Actocortin®) Frederiksberg Chemiske Fabriker A/S, Copenhagen, Denmark) dissolved in distilled water was added to serum at 37° C prior to the addition of KRG in order to avoid precipitation of calcium phosphate.

Serum

Blood from rats of the CDF strain (Charles River Breeding Labs. Wilmington, Mass., U.S.A.) conventionalized for more than 10 generations, was collected by heart puncture, and serum was stored at –20 C.

Determination of Phagocytosis and Release of Label from Bacteria into Medium

was carried out as previously described (6). The opsonic activity of serum was studied by measuring the ability of glass-adherent PMN to engulf ³²P labelled *E. coli* suspended in media containing 10 per cent of the serum to be tested. The uptake of radioactivity into PMN was expressed as cpm per mg cell protein.

Bactericidal activity of serum was measured indirectly using release of label into the medium as a parameter of the bactericidal effect of the 10 per cent serum present.

The bacterium was the same strain (X7) of *E. coli* as the one used in previous studies (1, 6). The number of bacteria suspended in the medium was 10⁹ per ml.

Pre-incubation

The drug was pre-incubated separately with either serum, bacteria or PMN. As stated in the text, the experimental conditions were varied in sequence. In some experiments the dose response was studied. In other experiments the influence of different temperatures or periods of pre-incubation was studied.

Statistical Analysis

was carried out by use of the two samples ranks test of Wilcoxon-White (5, 7).

RESULTS

Pre-incubation of Methylprednisolone with Serum

A. Effect on Release of Label from Bacteria (Bactericidal Activity)

1. *Dose of drug.* The effect of various concentrations of methylprednisolone on the rate of release of label from bacteria, induced by 10 per cent serum pre-incubated for 30 min at 37 C with the drug, is shown in Fig 1. The two highest concentrations used (1 and 2 mg per ml) reduced the rate of release significantly ($p < 0.01$).

2. *Time of pre-incubation.* Serum was pre-incubated with 2 mg methylprednisolone per

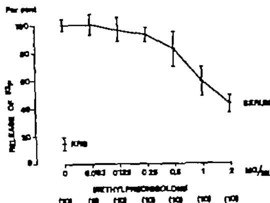


Fig 1. Release of label from ³²P-labelled *E. coli* suspended in medium without serum (KRG) or KRG with serum (10 per cent) pre-incubated with methylprednisolone at 37 C for 30 min. The rate of release in the presence of serum without methylprednisolone is set as 100 per cent. I = ± 1 SD. Number of observations is indicated in brackets.

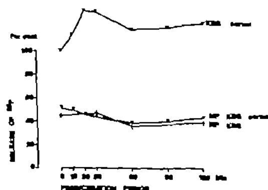


Fig 2. Influence of pre-incubation time on the blocking effect of methylprednisolone ((MP) 2 mg per ml) on the bactericidal activity of serum after 15 min incubation at 37 C. Release of ³²P-labelled *E. coli* in the presence of different media pre-incubated at 37 C for 0–120 min, i.e. KRG + MP, KRG + serum + MP. The rate of release in the presence of serum without MP and with no pre-incubation is set as 100 per cent.

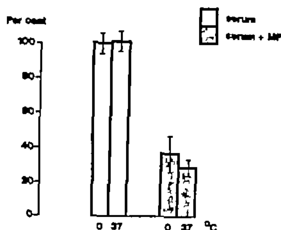


Fig 3 Influence of pre-incubation temperature on the blocking effect of methylprednisolone (MP) on bactericidal activity of serum against ³²P labelled *E. coli*. Pre-incubation of serum (10 per cent) with and without methylprednisolone (2 mg per ml) for 120 min at 0° and 37 C. The rate of release of label from bacteria in the presence of serum pre-incubated at 0° C without the addition of methylprednisolone, is set as 100 per cent. I = ± 1 SD

ml for different periods of time (0–120 min) The results of a typical experiment are illustrated in Fig 2. The addition of methyl prednisolone reduced release of label in the presence of serum to that in the absence of serum. The reduction appeared to be of the same degree whether a pre incubation period of 0 min or one of 120 min was used.

3 *Temperature of pre incubation* When serum was pre-incubated with methylprednisolone (2 mg per ml) at 0 C or 37 C for 120 min, the rates of release were both reduced to the level observed when *E coli* were suspended in KRG without serum (Fig 3) ($p > 0.10$)

B Effect on Uptake of E. coli *by PMN (Opsonic Activity)*

The opsonic activity of the serum present was estimated by its ability to induce uptake of ³²P labelled *E. coli* by PMN The effect of pre-incubating serum with methylprednisolone at 37 C for 30 min is shown in Fig 4 At a concentration of 1 mg per ml or higher the rate of uptake was reduced to that observed in the absence of serum (KRG) while at 0.5 mg per ml or lower concentrations the rate of uptake was considerably higher

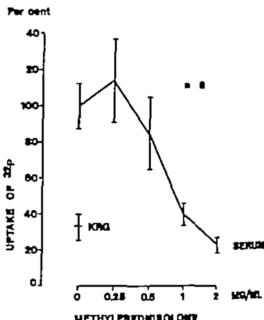


Fig 4 Uptake of ³²P-labelled *E. coli* by PMN in the absence (KRG) or in the presence of 10 per cent serum pre-incubated at 37 C for 30 min after addition of various amounts of methylprednisolone. The rate of uptake in the presence of serum without methylprednisolone is set as 100 per cent. I = ± 1 SD (n = number of observations)

Pre-incubation of Methylprednisolone with PMN

The effect of pre-incubating PMN with various amounts of methylprednisolone (stated as mg per ml of the pre incubation mixtures) is shown in Fig 5 After preparing monolayers of PMN the KRG medium was exchanged with one containing methylprednisolone in KRG and pre incubated for 30 min at 37 C. This medium was removed by decanting the tubes. Subsequently the uptake of label by PMN was measured following incubation for 15 min at 37 C in a suspension of labelled *E. coli* in KRG with serum. The rate of uptake by PMN pre-incubated in KRG without the addition of methylprednisolone, was set as 100 per cent. Pre-incuba-

DISCUSSION

The bactericidal and opsonic activity of serum could easily be measured in the *in vitro* model used (6). In this model, steroids might influence each of three factors, i.e. 1) serum factors, 2) PMN or 3) the microbe used. All three factors were evaluated in the present study.

No significant effect on the sensitivity of *E. coli* to serum was found in the present study.

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As indicated by the present results, high concentrations of methylprednisolone (1 mg per ml and higher) impaired the bactericidal and opsonic functions of rat serum factors against *E. coli*. This blocking effect was independent of pre-incubation (Fig 2). At this point the present results did not support the results obtained by *Gewurz et al.* (3) who found that a pre-incubation period was required to achieve inhibition of guinea-pig complement by hydrocortisone in a haemolytic system, using sheep red cells sensitized with rabbit antibody. In addition, the present results did not support their observation that methylprednisolone in equivalent quantities was more effective than hydrocortisone in equimolar concentrations.

A blocking of humoral factors might be obtained by lower concentrations of the drug *in vivo* provided that a splitting of the compound was necessary to release the active component, and that this breakdown of the drug might occur more easily *in vivo* than *in vitro* (2). Therefore, different compounds of hydrocortisone were tested. In Actocortin® hydrocortisone is loosely bound to phosphate and is immediately split off when the compound comes into contact with serum. The blocking effect of this compound was equal to that of hydrocortisone succinate or methylprednisolone succinate in equimolar concentrations. The concentrations of the latter drug required to impair the serum functions studied were at least 25 times larger than that obtained in clinical conditions (2).

It seems unlikely therefore, that the effect on humoral defence factors observed in the present study should be of importance in an attempt to explain the infection-enhancing ability of steroids. Further studies on their influence on the formation of antibacterial serum factors and on white blood cells are in progress.

This study was supported by grants from *Norsk Medisinaldepot* and *Norges Almenvitenskapelige Forskningsråd*.

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Acta path. microbiol. scand. Sect. B 82 701-706 1974

ANTIMYCOBACTERIAL ANTIBODIES IN SERA FROM PATIENTS THROUGHOUT THE CLINICO-PATHOLOGICAL DISEASE SPECTRUM OF LEPROSY

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Sera from one hundred and thirty-nine leprosy patients, clinico-pathologically classified into seven groups, were examined for precipitating antimycobacterial antibodies by double diffusion in gel analyses. As the source of antigen, *Mycobacterium duvalii* tended to be superior to *Mycobacterium bovis* (BCG) and revealed precipitins in fifty-seven of the sera. Throughout the leprosy spectrum, the proportion of precipitin-positive sera increased from the tuberculoid to the lepromatous end giving a maximum of 73.6 per cent positive sera in the polar lepromatous (LL) group. Similarly the number of precipitin lines also increased and in some lepromatous sera five lines were deciphered. The presence of precipitins was closely related to the density of *Mycobacterium leprae* in the skin of the patients. This suggests that the antigenic load is the main factor in determining the antibody formation in leprosy

It has become increasingly clear that the cell-mediated immune response (CMI) to *Mycobacterium leprae* is the main factor in determining where the patients are placed in the disease spectrum of leprosy (8) On the other hand, since *M leprae* is an intracellular parasite, the humoral antibodies produced are not considered to have any direct influence on the bacillary load and the resulting clinico-pathological picture (20) The frequent finding of antimycobacterial antibodies in patients with lepromatous leprosy (1 21) in whom *M leprae* apparently multiply without interference of host defence mechanisms, is in full accordance with this view

There is, however strong evidence that the antibodies formed are not merely innocent bystanders. Immune complexes appear to be responsible for hypersensitivity reactions (erythema nodosum leprosum) in highly bacilliferous patients (7 18 21) Moreover it has also been suggested that the antibodies formed may have an adverse effect by contributing to the state of CMI unresponsiveness to *M leprae* in lepromatous leprosy patients (17 20)

Hence, the humoral immune response to *M leprae* antigens must be considered and, in the present study sera from patients throughout the leprosy spectrum have been examined for a presence of precipitating antimycobacterial antibodies. Such precipitins were found with increasing frequency as one moved from the 'high resistant' (tuberculoid) towards the 'low resistant' (lepromatous) end of the spectrum.

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DISCUSSION

The bactericidal and opsonic activity of serum could easily be measured in the *in vitro* model used (6). In this model, steroids might influence each of three factors, i.e. 1) serum factors, 2) PMN or 3) the microbe used. All three factors were evaluated in the present study.

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It seems unlikely therefore, that the effect on humoral defence factors observed in the present study should be of importance in an attempt to explain the infection-enhancing ability of steroids. Further studies on their influence on the formation of antibacterial serum factors and on white blood cells are in progress.

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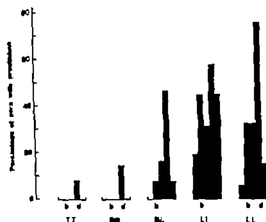


Fig 2 Percentage of TT BB, BL, LI and LL sera showing the various precipitins to *M duvalii* antigens.

leprosy sera. The combination of undiluted serum and antigen concentrations (per ml) corresponding to the release from 263 mg (wet weight) *M duvalii* and from 548 mg BCG was found to give the best results. These concentrations were therefore used for the study

Fifty-seven of the 139 sera from leprosy patients showed precipitin lines, while all of the sera from fifteen patients with tuberculosis and from twelve household contacts of leprosy patients were negative for precipitins. The antigen preparations from *M duvalii* and BCG both produced a maximum of five lines. These five precipitins were designated as *a*, *b* *c* *d* and *e* where *a* refers to the line closest to the serum well and *e* the line closest to the antigen well. One of the sera, showing all five precipitins, was used as reference serum to characterize the others. Of the lines produced by BCG and *M duvalii*, two lines showed identity one partial identity and one non-identity while the relation of the fifth precipitin was difficult to assess. When the two antigen preparations showed different numbers of precipitins, *M duvalii* invariably produced more lines than BCG Therefore, only results obtained with *M duvalii* antigens are presented below

Fig. 1 shows the percentage of sera in which precipitins were detected in each of the seven groups of leprosy patients. 73 6 per cent (39/53) of the LL sera showed precipitins, 62.5 per cent (10/16) of the LI sera, 46.2 per cent (6/13) of the BL sera, 14.3 per cent (1/7) of the BB and 7 7 per cent (1/13) of the TT sera. Precipitins could not be detected in any of the TT/BT and BT sera examined. The figure also shows the number of precipitating lines that could be deciphered. The positive TT and BB sera showed one precipitin line, the positive BL sera from one to three, while up to five lines could be identified with the LI and LL sera.

Among the five precipitin lines observed, precipitin *d* was most frequently demonstrated and was found in 56 out of the 57 positive sera. Precipitins *b* *c* *e* and *a* were found in 25 24 16 and 6 sera, respectively The distribution of the various precipitins throughout the spectrum is illustrated in Fig 2. The LL sera showed the highest percentages of precipitins *c* and *d* while precipitins *a*, *b* and *e* were most frequently encountered in the sera from the LI patients.

Fourteen of the 53 LL sera examined were obtained from patients with erythema nodosum leprosum (ENL) Twelve of these sera, i.e. 85 7 per cent, showed precipitins, while

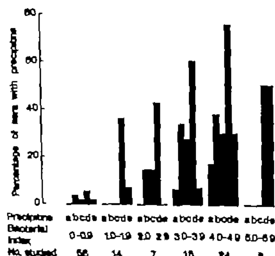


Fig 3 Relationship between the density of bacteria in the skin of leprosy patients and the presence of the various circulating antibodies to *M duvalii*.

the figure for the remaining LL sera was 69.2 per cent. On the other hand, the maximum number of precipitins was lower in the ENL sera than in the sera from LL patients without immune complex syndromes. Precipitins *a* and *e* were not detectable in any ENL serum, and a maximum of only three lines could therefore be deciphered.

Whether or not the patients had received treatment did not seem to influence the presence of precipitating serum antibodies. When evaluating the results separately for the 66 untreated and the 55 treated patients, the percentage of sera showing precipitins was about the same among treated and untreated BL, LI and LL patients. The only positive TT serum was obtained from a patient who had received treatment for some time, while the positive BB serum came from an untreated case.

Fig 3 shows the percentage of sera with the various precipitins when 120 sera were grouped according to the bacterial index of the patients. When the bacillary load was low precipitins were rarely demonstrated. As the number of bacilli in the skin increased, so generally did all the precipitins. However the two sera from patients with the highest bacterial indices showed only one line each.

DISCUSSION

It seems reasonable to conclude that the precipitating antimycobacterial antibodies demonstrated in a proportion of leprosy sera resulted from the infection with *M leprae* The sera were obtained from patients without clinical evidence of other mycobacterial infections, and moreover if another mycobacterium had been the immunizer one would also have expected to find precipitins in some of the sera from tuberculous patients and household contacts of leprosy patients.

Ideally *M leprae* antigens should have been used for testing the humoral immune response in leprosy But, as the bacillus cannot be grown on artificial media, enough material for double diffusion in gel studies cannot be obtained at present. Since antigens from presumably related organisms were used, the study does not give information about antibodies produced against *M leprae* antigens not shared by the two related species.

The finding that at least one of the five precipitin lines produced against *M duvalii* and BCG respectively showed non-identity shows that six different serum antibodies stimulated by *M leprae* were identified. This could indicate that the leprosy bacillus, in addition to the antigens common for all three species, has antigens which it shares only with *M duvalii* and others shared only with BCG In view of results from studies using other immunological methods which indicate a close relationship between *M leprae* and *M duvalii* (6) It is interesting that *M duvalii* precipitates with antibodies to *M leprae* more easily than BCG However a difference in the release of the relevant antigens into solution, possibly related to differences in the susceptibility to ultrasonic disintegration (*Closs O* personal communication) could also explain the findings.

The study shows a gradual increase in

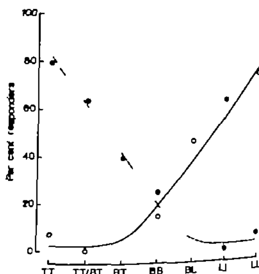


Fig 4 Comparison between the *M leprae*-induced lymphocyte transformation (from (1)) ●—— and the demonstration of circulating antimycobacterial antibodies, ○—— throughout the leprosy spectrum.

precipitin-positive sera and in the number of demonstrable precipitins towards the lepromatous end of the leprosy spectrum. This means that antimycobacterial antibodies were rarely demonstrated in sera from patients having few and well-defined lesions, but their presence increased as the lesions became multiple and the disease progressed. Thus, with decreasing clinical and histological evidence of resistance, the frequency of sera showing antibodies stimulated by the causative micro-organism increases.

This predominant restriction of circulating antimycobacterial antibodies to patients with bacilliferous progressive forms of the disease is, in the main, in agreement with other gel diffusion studies of the humoral immune response in leprosy (9 11 16). A detailed comparison between these previous studies and the present results, however is difficult, since the now widely accepted spectral concept of leprosy (13) formed the basis of the patient classification only in the present study. Other differences which would also complicate the comparison are related to the mycobacterial species used, the method of antigen preparation and the geographic origin of the sera tested.

The close association between density of *M. leprae* in the skin of the patients and the presence of serum precipitins (Fig 3) seems to indicate that the antigenic load is a main factor in determining the antibody production. The fact that it takes more than five years of continuous therapy to render most lepromatous patients bacteriologically negative (5 19) may explain why the percentage of positive sera was about the same in groups of treated and untreated patients. Most of the treated patients had received treatment for only a few weeks or months, and thus, there is necessarily no discrepancy between our findings and the gradual fall in antimycobacterial antibody titre with treatment reported by *Rees et al.* (11). However the finding of only one precipitin in each of the sera from the two patients with the highest bacterial indices could indicate that factors other than bacillary load are involved in the regulation of antibody formation. Examinations in a highly resolving immunochemical system (2) might provide further information on this point, and also on the differences observed in demonstrable precipitins in sera from lepromatous patients with and without immune complex syndromes (ENL).

The humoral immune response apparently contrasts sharply with the CMI response to *M. leprae in vivo* and *in vitro* (8). This is illustrated in Fig. 4 where the frequency of precipitin-positive sera in patients of each group is compared with the proportion of patients whose lymphocytes undergo blastoid transformation when stimulated with *M. leprae in vitro*. (This method is a good correlate of CMI in leprosy (8)). Throughout the leprosy spectrum there is an approximate inverse relationship between the percentage of responders to the test by which humoral immunity is measured and the test by which cell-mediated immunity is mainly measured.

While the antibody formation may be a function of the bacillary load, the reason for the gradual fall in CMI responsiveness to *M. leprae* towards the lepromatous end of the spectrum remains unknown. The extreme findings in lepromatous leprosy have raised the key question whether anti-*M. leprae* antibodies are directly involved in the specific suppression of the CMI response to *M. leprae*. A mechanism anologous to immunological enhancement has been postulated (17 20) but it has not been possible to show any blocking effect of lepromatous sera on T-cell responses to *M. leprae in vitro* (4). Findings in the present study also tend to point away from a direct suppression of CMI by antibodies alone as two of the precipitin positive sera, one from a TT patient and another from a borderline patient in reversal reaction (5) came from individuals showing strong CMI responses to *M. leprae*. Thus, there is at present no evidence in favour of a mechanism implying that humoral antibodies at all times take part in the suppression of the CMI response to *M. leprae*. It might, therefore be more reasonable to consider the im-

munological defect of lepromatous leprosy as a consequence of a deviation of the immune response into humoral immunity How ever antibodies may also be directly involved in the suppression of CMI if such a mech anism is operating (10 22)

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IMMUNE RESPONSIVENESS TO *MYCOBACTERIUM LEPRAE* OF HEALTHY HUMANS APPLICATION OF THE LEUCOCYTE MIGRATION INHIBITION TEST

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Immune responsiveness to *Mycobacterium leprae* was studied, by the method of leucocyte migration inhibition, in ninety healthy adults allocated into four groups according to previous contact with leprosy patients. Groups working or living in close relationship with leprosy patients responded significantly more strongly to *M. leprae* than a group without such contact. With a selected concentration of *M. leprae* 71.2 per cent of medical attendants dealing with leprosy patients, 22.2 per cent of administrative staff of a leprosy hospital, and 50 per cent of household contacts of leprosy patients showed migration indices <0.800, but none of the group without known contact with leprosy patients showed indices below the threshold value. Since the inhibition of migration to BCG was similar in all groups, and no evidence was found that other mycobacteria had provoked the positive responses elicited by *M. leprae* the above figures appear to represent individuals immunologically stimulated with *M. leprae* itself. The study therefore, showed that the method of leucocyte migration inhibition may be used as an assay for specific detection and enumeration of immune responses mounted by *M. leprae* The results lend strong support to the view that leprosy bacilli are frequently transmitted from patients to contacts. The introduction of *M. leprae* into the human body is, however rarely accompanied by development of clinical signs of leprosy

A main problem impeding progress in the epidemiological understanding of leprosy has been the lack of methods for detection of individuals who although exposed *Mycobacterium leprae* do not develop the disease clinically. The conception that mechanisms of innate or acquired immunity may frequently prevent the infection from becoming manifest (20) has, therefore, remained merely a supposition.

However the establishment of *in vitro* methods for measuring immune responses to *M. leprae* (7 8, 12) has provided instruments possibly suitable also for epidemiological studies. Thus, studies with the lymphocyte transformation test have shown that a large proportion of people with occupational or household contact with leprosy patients reveal immunological evidence of exposure to *M.*

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leprae (6, 9) From these studies it was deduced that leprosy bacilli are frequently transmitted from patients to contacts, and that most individuals exposed acquire effective immunity to the infection.

In the present paper results of a similar investigation on the inhibition of leucocyte migration elicited by *M leprae* are reported. Technical emphasis was placed in establishing a method with high specificity to *M leprae* as cross-reactivity with BCG and *Mycobacterium tuberculosis* has complicated the interpretation of the lymphocyte transformation findings. The results obtained provide strong additional evidence in favour of the view that people dealing with infectious leprosy patients are likely to become exposed to *M leprae*

MATERIALS AND METHODS

Test Subjects

Ninety healthy adults participated in this study None of them had any previous history of clinical mycobacterial infection. They were considered in four main groups according to their contact with leprosy patients.

Seventeen of those examined claimed no known contact with leprosy patients. This group is hereinafter called the non-contact group. Ten of these non-contacts had arrived in a leprosy endemic area, i.e. Ethiopia, less than one month prior to the examination. The other seven had lived f from one to twenty years in countries where leprosy is endemic. Twelve out of the seventeen were BCG-vaccinated, while the other five claimed exposure to patients with tuberculosis. All except one in this group came originally from countries outside Africa.

Fifty two of the participants were medical personnel working in various departments of leprosy hospitals or in leprosy control programmes All had regularly been in contact with leprosy patients *for periods ranging from 2 months to 19 years.* Exact data concerning BCG-vaccination and exposure to *M tuberculosis* was difficult to obtain However at least half of the group were BCG-vaccinated, and it is very likely that majority had been in contact with patients with tuberculosis. Threequarters of these medical attendants were African.

A third group investigated consisted of nine leprosy hospital (ALERT) staff members, not dealing with patients These nine individuals, mainly on the administrative staff, had been in their jobs for from 2 to 12 years They had all, in their daily work, been in the vicinity of leprosy patients, but rarely in close contact. Only one reported to have been BCG-vaccinated. Eight of these nine non-medical staff were Ethiopians.

Finally Twelve people living in the same house as leprosy patients were examined. Six of these were contacts of a borderline leprosy patient (BT BB or BL) three were contacts of a lepromatous leprosy case (LL) while each of the remaining three lived together with several patients with various forms of the disease (See *Ridley & Jopling* (19) for the detailed clinical and histo-pathological classification of leprosy patients) The length f contact varied from 1½ to 30 years. Two of the household contacts, who were all Ethiopians, claimed to have been BCG-vaccinated. No reliable information on exposure to patients with tuberculosis could be obtained.

Antigen

M leprae bacilli were obtained from skin biopsies from patients with lepromatous leprosy The preparation of bacilli from tissues was according to *Godal et al.* (7) Materials harvested from several biopsies were pooled and stored in suitable portions at −70 C until used. BCG (Glaxo Lab. Ltd., Greenford, Middlesex, England) obtained in lyophilized ampoules, was suspended in tissue culture medium, counted, and stored in the same way as *M leprae*.

Tissue Culture Medi m

Eagle s medium (BDH Chemicals Ltd., Poole, England) was used with the following ingredients added: 2.64 g NaHCO per litre 100 units penicillin per ml, 5 per cent horse serum and 5 per cent normal human serum from persons without known exposure to leprosy patients In previous study this combination of horse and human serum in the medium was found to give stronger inhibition of leucocyte migration than horse serum alone (8) When the migration assay was performed the starting pH of the medium was adjusted to about 7.3.

Leucocyt Migration I hibition

A *modified ersion* of the technique of *Söborg & Bendixen* (21) was used. Routinely heparinized blood was allowed to sediment by gravity at 37 C for 60 to 90 minutes. However the blood samples from three household contacts and one medical attendant were collected *in the field*, and sedimentation took place during transportation for two hours at about 25 C. The leucocyte-rich plasma was pipetted-off and centrifuged (150 *g for* 10 minutes) The cell pellet, predominantly of leucocytes, was washed once and then resuspended in tissue culture medium. The volume of medium

added was adjusted to produce cell columns of approximately 3 mm length in microcapillary tubes (20 µl Drummond microcaps, Shandon Scientific Ltd., Willesden, London) introduced for this purpose by *Federlin et al.* (5). The cell suspension was then sucked up into the capillary tubes previously siliconized and sealed at one end. The capillaries were centrifuged at 600 *g* for five minutes and cut at the cell-fluid interphase, care being taken to avoid plugging by thrombocytes. The cell-containing parts were fixed in the bottom of plastic chambers (Universal Mekaniska Verkstad, Enskede, Sweden) two in each. Three chambers were filled up with tissue culture medium only and served as controls. *M. leprae* or BCG were added to the medium in the other chambers. For each concentration (in the final experiment 5×10^7 and 7.5×10 bacilli/ml) of each species three chambers were regularly set up. Incubation of the cultures and quantitation of migration was performed as previously described (8). Results were expressed as migration index:

$$\frac{\text{Average area of migration with antigen}}{\text{Average area of migration without antigen}}$$

Statistical Analyses

Standard deviation was used to express variance. Differences in the results between the various groups were analysed by the Wilcoxon two sample test (14). The significance of difference in the proportion of responders in the different categories of participants was measured by the chi-square test, using graphs (prepared by Miss *M. V. Mussett*, Statistical Service Section, National Institute for Medical Research, London) based on Mainland tables (10). The degree of relationship between the responses to *M. leprae* and the BCG was measured by the Kendall rank correlation coefficient (14).

RESULTS

In initial screening experiments it was found that concentrations of 5×10^7 and 7.5×10^7 *M. leprae* per ml frequently inhibited the migration of leucocytes from contacts of leprosy patients, but did not noticeably interfere with the migration of leucocytes from non-contacts. Such a distinction was not obtained with lower or higher concentrations. Thus, the use of 2.5×10 *M. leprae*/ml hardly resulted in inhibition in any of the cultures, while the inhibition induced by 1×10^8 bacilli/ml was not decisively related

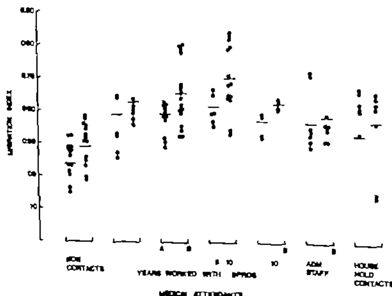


Fig. 1. M. leprae-induced inhibition of leucocyte migration in people without known contact with leprosy patients, medical personnel dealing with leprosy patients, administrative staff in a leprosy hospital and household contacts of leprosy patients.

(A) Antigen concentration = 5×10 *M. leprae*/ml

(B) Antigen concentration = 7.5×10^7 *M. leprae*/ml.

Mean values are shown as horizontal lines.

TABLE 1 *BCG-induced Inhibition of Leucocyte Migration Expressed as Migration Index (MI) of Groups of Persons with Various Degree of Contact with Leprosy Patients*

	5×10^7 bacilli/ml		7.5×10^7 bacilli/ml	
	No.	Mean MI ± SD	No.	Mean MI ± SD
Non contacts	17	0.766±0.120	17	0.692±0.106
Medical attendants <1 year	8	0.779±0.103	10	0.716±0.109
Medical attendants 1–5 years	15	0.764±0.114	15	0.676±0.111
Medical attendants 5–10 years	11	0.752±0.166	12	0.708±0.157
Medical attendants >10 years	8	0.818±0.128	8	0.756±0.119
Administrative staff	5	0.809±0.139	6	0.767±0.176
Household contacts	12	0.788±0.117	12	0.[illegible]62±0.132

to contact with leprosy patients. The study was therefore undertaken with 5×10^7 and 7.5×10^7 *M leprae*/ml and similar concentrations of BCG were used.

The *M leprae* induced inhibition of leucocyte migration in the various groups of participants is shown in Fig 1

Among individuals without previous contact with leprosy patients the migration index varied from 0.839 to 1.130 with the antigen concentration of 5×10^7 *M leprae*/ml and from 0.818 to 1.093 with 7.5×10^7 bacilli/ml. The corresponding mean values were 0.962±0.080 and 0.911±0.075 People living in leprosy endemic areas did not respond more strongly than those who had just arrived from non-endemic areas.

The medical attendants were allocated into sub-groups according to the length of time they reported to have been working with leprosy patients. In general they responded more than the non-contacts, but a wide range of responses was also observed within this group. For the whole group the mean migration index was 0.808±0.097 for the lower and 0.738±0.108 for the higher concentration of bacilli. A comparison between the subgroups showed that people who had worked in the field of leprosy for from 5 to 10 years responded on the average most strongly while those who had been leprosy workers for more than 10 years showed the weakest responses. The difference between the groups was, however small and not statistically significant ($p>0.05$) Staff in outpatient clinics frequently showed stronger responses than staffs in wards and service departments like laboratory and physiotherapy There was no apparent relationship between the degree of migration inhibition and social status, country of origin or place of living of the participants.

In the administrative staff group the mean *M leprae* induced migration inhibition, expressed as migration index, was 0.835±0.094 for the lower and 0.816±0.086 for the higher antigen concentration. The inhibition of migration did not seem to be related to length of time of hospital employment.

The corresponding figures for the household contact group were 0.874±0.123 and 0.833±0.154 The spread of the results was more pronounced among household contacts than in any of the other groups ranging from 0.733 to 1.068 with the lower and from 0.547 to 1.060 for the higher antigen concentration.

The non-contact group response was significantly weaker than either the medical attendants ($p<0.01$) administrative staff ($p<0.01$) or the household contacts ($p<0.02$) to the lower concentration of *M leprae* With the higher concentration a statistically significant difference was found between non-contacts and medical attendants ($p<0.01$) non-contacts and administrative staff ($p<0.02$) and between medical attendants and administrative staff ($p<0.05$)

Of the four main groups of participants the non-contacts responded on the average most strongly to both concentrations of BCG

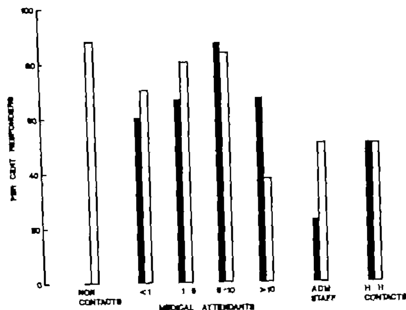


Fig 2 Percentage of non-contacts, medical attendants, administrative staff and household contacts showing migration indices <0.800 to *M leprae* (■) and to BCG (□)

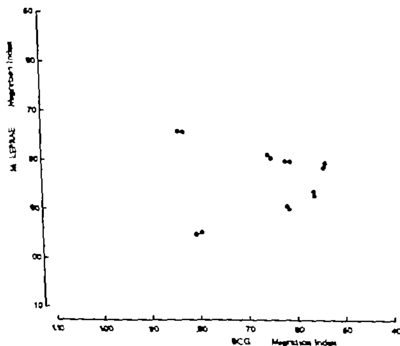


Fig 3 Comparison between the inhibition of leucocyte migration to *M leprae* and BCG of individuals without known contact with leprosy patients (○) and medical attendants dealing with leprosy patients (●)

(Table 1), but the difference in relation to the other groups was small and not statistically significant. The inhibition elicited by BCG was stronger in all groups than that of the corresponding concentration of *M leprae*

Figure 2 shows the proportion of individuals in the different groups having migration indices <0 800 to the higher (7.5×10^7 bacilli/ml) concentration of *M leprae* and BCG None of the non-contacts showed responses to *M leprae* stronger than the threshold value while the total figure for the medical attendants was 71.2 per cent responders, ranging from 86.7 per cent among those who had worked with leprosy patients for from 5 to 10 years to 60 0 per cent among those with less than 1 year's leprosy work. In the administrative staff and household contact groups, respectively 22.2 per cent and 50 0 per cent of the migration indices were <0 800 The difference in proportion of responders between the non-contact and the medical attendant as well as the household contact group was statistically significant ($p<0\,01$)

The percentage of responders to BCG showed a different pattern. Thus, 88.2 per cent of those without known contact with leprosy patients had a BCG induced migration index below 0 800 compared to 71 1 per cent among medical attendants and 50 0 per cent in the administrative staff and household contact groups. The proportion of responders was not significantly different between any of the groups.

In Fig 3 migration indices obtained with the higher concentrations of *M leprae* and BCG in non-contacts and medical attendants are plotted against each other The rank correlation coefficient for the non-contact group was 0 06 and for the medical attendant group –0 12. Similar low coefficients of correlation were found in the administrative staff (–0 20) and household contact groups (–0.27) Thus, the relationship between the responses to *M leprae* and BCG was poor and not statistically significant in any of the groups.

DISCUSSION

In this study groups of people in close contact with leprosy patients were found to respond significantly more strongly to *M leprae* in the leucocyte migration inhibition test than people without such contact. This difference is unlikely to be due to immunization with *M tuberculosis* BCG or environmental mycobacteria, since a) all non-contacts were either BCG-vaccinated or exposed to tuberculous patients, b) the migration inhibition induced by BCG *in vitro* was as strong among non-contacts as in the other group and c) there was no obvious correlation between the response to *M leprae* and social situation, place of domicile or country of origin of the participants. I, therefore, conclude that the stronger responses to *M leprae* among people dealing with leprosy patients appear to be caused by previous immunological stimulation with *M leprae* itself

The initial experiments indicate that the concentration of *M leprae* used is critical in distinguishing responses provoked by *M leprae* from responses which also may be due to sensitization with other mycobacteria. A good distinction was, however achieved with both the concentrations used in the study The high specificity of the method with these selected amounts of antigen, shows that the leucocyte migration assay may be rendered usable for specific detection and enumeration of immune responses to *M leprae*

Since the most strongly responding non-contact had a migration index of 0.818 it seems reasonable to suppose that the maximal inhibition caused by immunization with other relevant cross-reacting mycobacteria would be of about that range It, therefore, appears likely that people with migration indices lower than the chosen threshold of 0.800 have acquired a specific immune response to *M leprae* No extra-human source of *M leprae* is known, and the study does, therefore, provide strong additional evidence in favour of the view that leprosy bacilli are frequently transmitted from patients to contacts (6, 9)

The *M leprae*-exposed individuals detect-

ed here are restricted to those who, subsequent to exposure, have raised an immune response to *M. leprae* detectable by the assay established. The total number of *M. leprae* exposed persons may, therefore, exceed the number of people found to show immunological evidence of exposure. There may be various factors influencing the ratio between these two figures. Thus, *Godal & Negassi* (9) have suggested that super-exposure decreases the proportion detectable by immunological methods. Some caution should, therefore, be used when estimating from the figures obtained the risk of exposure in the special situations studied. However, in the main there ought to be a good correlation.

The study was not designed to throw light on which particular patients spread *M. leprae*. Accumulating evidence suggests, however, that the main source for the distribution of *M. leprae* is through the nose of untreated and inadequately treated lepromatous leprosy patients (15, 16, 18). Transmission of *M. leprae* to new hosts preferably takes place, therefore, where this category of patient appears, and is probably reflected in the strong response to *M. leprae* among staff of out-patient departments. Some of these bacilliferous patients are long regarded as healthy because they have very minor clinical manifestations of the disease. Such patients are likely to shed a vast number of bacilli which are unwittingly transmitted to contacts, and this could be the reason why many leprosy patients do not know their source of infection (1). Hence anyone living in a leprosy endemic area incurs the risk of becoming exposed to *M. leprae* as suggested in the data of *Godal & Negassi* (9). The present finding that none of the seven non-contacts living in leprosy endemic countries revealed immunological evidence of exposure, may merely indicate that the likelihood is low for the expatriot societies concerned.

The prevalence of leprosy among people dealing with leprosy patients (1, 5, 13, 20) contrasts sharply with the prevalence of exposure found in the present study. Although the true prevalence of leprosy among leprosy workers in Africa may be higher than the 1 per cent figure suggested by *Cochrane* (2), it is obvious that, in an endemic situation, a very low proportion of individuals exposed to *M. leprae* develop clinical signs of leprosy.

This raises the question: Why do those who show immunological evidence of exposure not contract the disease clinically? It could be that most new hosts are receiving non-viable bacilli only. The knowledge about the portal of entry and method of spread of *M. leprae* is too scanty to seriously consider this possibility. It also remains unknown whether non-viable organisms are able to provoke an immune response detectable by the *in vitro* assays established. However, the ability of lepromin (autoclaved *M. leprae*) to induce dermal delayed sensitivity to itself (4, 17) may indicate that non-viable leprosy bacilli evoke a cell-mediated immune response in man.

The alternative explanation of the great difference between exposure and prevalence rate would be that the defence mechanisms of most new hosts control the infection before clinical manifestations develop. *Skinsnes* (20) has suggested that non-specific, mainly innate, factors of immunity regularly prevent *M. leprae* from gaining foothold in new hosts. Demonstration of acquired immune responses to *M. leprae* shows that bacilli or bacillary material pass this first line of defence. However, this does not necessarily throw light on the relative importance of innate and acquired immunity as protective mechanisms.

The similar results obtained with both the leucocyte migration inhibition test and the method of lymphocyte transformation (6, 9) point to a stimulation of the cell-mediated immune system. Whether exposure to *M. leprae* also regularly leads to production of anti-*M. leprae* antibodies is not known. Double diffusion in gel analyses did not reveal antimycobacterial antibodies in sera from family contacts of leprosy patients (11). A further evaluation of the humoral immune response induced may, however, be important for increased understanding of the circumstances that turn a few *M. leprae*-exposed

individuals into patients. The antibodies formed could act in concert with the cell mediated immune response to the benefit of the host, but interactions between humoral and cell-mediated immunity might also lead to suppressed resistance (22)

Thanks are due to the volunteers who donated blood for the study to Miss *Monica Lefgren* and Mrs. *Monica Hyde* for skilful technical assistance, and to Dr *T Godal* and Dr *M Harboe* for valuable comments on the manuscript.

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DETERMINATION OF ANTISTREPTOLYSIN O BY REVERSED SINGLE RADIAL IMMUNODIFFUSION

ANDERS FASTH

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A diffusion-in-gel micromethod for quantitation of ASO is presented. The method is compared with conventional dilution techniques. Non-reduced ASO and sheep erythrocytes were mixed in an agarose gel. Around holes filled with sera under test zones of inhibited hemolysis appeared when the ASO was reduced. The intra- and interplate variation were 5.5 and 28 ASO units The correlation coefficient between the method presented and that of *Cabau* & *Badin* was 0.938.

The first serological test for antistreptolysin O (ASO) was described by *Todd* (11) who also demonstrated the occurrence of antistreptolysin in sera from patients with various streptococcal diseases, glomerulonephritis, and rheumatic fever The serological tests for ASO are based on specific binding of the antibodies to streptolysin O with an inhibition of the hemolytic property as a result. The amounts of ASO can be estimated by dilution of the sera. A number of modifications of the original technique by *Todd* have been published (for references see (8)) It was observed early that the serum lipids mainly cholesterol function like non antibody streptolysin O inhibitors (6) The inhibitory effect of various steroids has since been investigated by *Badin* & *Barillac* (1) In our routine immunological laboratory a modification described by *Cabau* & *Badin* (3) has been used, where the lipids are precipitated with calcium chloride and dextran sulphate.

Zettervall (13) described a simple microtechnique using a diffusion-in-gel method. In his method hemolysis inhibition occurred around holes filled with sera containing ASO A modification of his technique has been developed for routine use and compared with the conventional technique used at the laboratory for many years.

MATERIAL AND METHODS

Sheep erythrocytes washed 3 times and a 5 per cent suspension in 0.15 M N Cl was prepared.

Streptolysin O in non-reduced form containing about 4 5 combining units/ml was obtained from the Swedish State Bacteriological Laboratory

Agarose (Miles Servac Ltd, Maidenhead, Great Britain) was dissolved in phosphate buffered saline, 0.15 M, pH 7 2, to a concentration of 1.2 per cent (w/) (stock solution)

Natriumpyrosulphite (Na_2S O) 10 per cent (w/v) was used as the reducing agent of the streptolysin O.

The samples were dalipidized as described by *Cabau* & *Badin* (3) by addition of an equal volume of a dextran sulphate-calcium chloride solution. After having been kept for 30 minutes at +4 C the samples were centrifuged at about 3000 rev/min. The supernatants were used at once or stored at –20° C until use.

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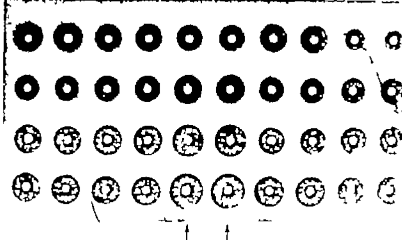


Fig 1 Estimation of the ASO contents in 16 sera. Each serum analysed twice. The samples are grouped in pairs from left to the right. Arrows indicate rows with serial dilutions of a reference standard serum. Diffusion time 15 hours, temperature +4 C.

A stock solution of agarose, 1.2 per cent, was prepared and kept in the refrigerator until use. The agarose was melted and then cooled to about 50 C and the sheep erythrocytes were added to a final concentration of 1 per cent. An appropriate amount of the streptolysin O was then poured into the agarose-erythrocyte mixture (45 C) Buffer was added to make a final agarose concentration of 0.6 per cent. The mixture was poured into a mould made of two glass plates (1 × 205 × 110 mm) separated by a U-shaped frame 1 mm thick and allowed to set. After gelation the top glass plate was gently slipped off and the frame was removed and row of holes, 2.5 mm in diameter and about 18 mm apart were punched out.

5 µl of the sera to be tested were deposited in the holes. The plates were placed in a moist chamber to allow the diffusion of the samples into the gel. The temperature and the time allowed for diffusion of the samples into the gel were aried to find the optimal conditions for quantitative estimation of the ASO The final step consisted of activation of the streptolysin O in the gel by soaking the plates with a solution of sodiumpyrosulphite t room temperature. Hemolysis of the erythrocytes not protected by the diffused antibodies was *complete within 10–15 minutes Filterpapers* soaked with sodium chloride 0 15 M, were placed over the gel surface. The filter papers were covered by a 1–1.5 cm layer of soft blotting paper and a light pressure was applicated. In about 10 minutes the gel contracted to thin film, and the filter papers were removed.

Circular zones of unlysed cells appeared (Fig 1) around the *holes filled with the sera tested* Two perpendicular diameters of the circles were measured under magnification (9 ×) in a table projector (Documator Lesegerät, Carl Zeiss, Jena)

RESULTS

Different temperatures and different periods of incubation of the samples were tried. Optimal conditions were obtained either with a diffusion time of about 6 hours in room temperature or *with over night* incubation, i.e. about 15 hours, in a refrigator A shorter diffusion time did not allow the high titer sera to diffuse enough. Lysis of the cells with incubation over night required a larger amount of streptolysin O This was also found when over night incubation was used for the agarose-blood-streptolysin mixture alone. Red cell suspensions in buffered saline seems to become somewhat resistant to streptolysin O on standing (5)

A batch of pooled sera containing 4400 ASO units as determinated by the method of *Cabau & Badin* (3) was used as a reference This standard was used for each plate in double serial dilutions. The method of *Fahey & McKelvy* (4) was used to express the correlation between the zones and their ASO content. The sum of two perpendicular diameters of the zones was plotted in a semilogarithmic diagram (Fig 2)

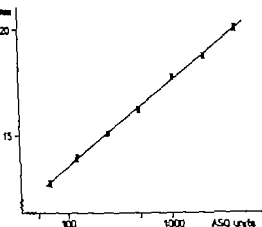


Fig 2 Results obtained by serial dilution of a reference standard serum containing 4400 ASO units. Each dilution is tested by double determinations Diffusion time 6 hours in room temperature

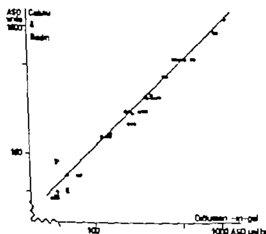


Fig 3 Correlation between the method of *Caban & Badin* and the diffusion-in-gel technique. 71 sera analysed with the two methods with single determination of each sample. The regression line is drawn (Y = 13 1 + 0.94)

Double determinations of 15 samples with a broad spectrum of titers were performed and the results compared with those obtained with the technique of *Caban & Badin* The correlation coefficient was 0.956 The equation of the regression line showed an intercept, on the ordinate, of 16 ASO units, which did not significantly differ from zero ($p > 0.01$)

The 15 sera were analysed on several occasions and the standard-deviation was calculated. The values of the intraplate deviation varied between 3.8 and 5.5 ASO units. The calculated interplate deviation was 26 ASO units.

To evaluate the method in routine work 71 sera were examined with the method of *Caban & Badin* and by the present diffusion-in-gel method but only single determinations were performed (Fig 3) The correlation coefficient was 0.936. The equation of the regression line showed an intercept, on the y-axis, of 13 ASO units, which value did not significally differ from zero ($p > 0.01$)

DISCUSSION

The method described uses the principles of the radial immunodiffusion techniques (4 9) in their reversed form, i.e. the antibody is permitted to diffuse in an antigen containing gel. *Vaerman et al.* (12) described the reversed system and found no difference between this method and the original radial immunodiffusion techniques.

The rapid oxidation of streptolysin O in air with reversible loss of its hemolytic power is utilized by method described. Thus the streptolysin in nonreduced form can be kept in a mixture of red cells and agarose during the time needed for the serum antibodies to diffuse into the gel. After the binding of ASO to the streptolysin O the reducing agent is added and zones of inhibition of hemolysis appear The zones are very well defined (Fig 1) and can easily be measured, most conveniently at a magnification of × 5–10.

The increasing resistance of the erythrocytes to streptolysin O and an irreversible oxidation of the lysin in course of time makes it necessary to measure the zones before equivalence is reached. That is in analogy with the technique of *Fahey & McKelvey* (4) measuring at the still expanding precipitin ring *Berne* (2) has closely analysed the methods of radial immunodiffusion and he showed that depending on the diffusion rate the linearity between log concentration of the diffusing antibody or antigen and the diam-

eter of the zone existed only for a couple of hours during the first 5 to 24 hours. That time has to be found empirically and for the method described two possibilities were found either 6 hours in room temperature or 15 hours in a refrigator (+4 C)

The results obtained with the immunodiffusion method agreed well with those obtained with the conventional methods. The interplate variation was 26 ASO units. A batch of pooled sera with a titer of 185 ASO units analysed by the method of *Cabau & Badin* in our routine laboratory on 40 consecutive days gave a standard deviation of 37 ASO units (about 20 per cent) Other authors (7 10) report the range of variation found by daily titration of the same serum to be in the order of 20–30 per cent.

One advantage of the method described is that it saves materials (plastic tubes, sheep cells and streptolysin O) The cost per sample when a whole plate is utilized for analysis, i.e. single determinations of 32 samples, is about one third of that of the serial dilution methods. A further advantage is the smallness of volume needed. The volume of blood necessary for the test can be obtained from a finger prick and easily collected in commercial 400 μl microtubes. This makes the method very useful for the investigation of serum from children.

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GRAFT-VERSUS-HOST REACTIONS MEDIATED BY A THYMUS-BONE MARROW COMBINATION SPLEEN CELLS, AND LYMPH NODE CELLS FROM AMYLOIDOTIC AND NONAMYLOIDOTIC MICE

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GVH-reactions were induced in (C3H × St/A) and (C3H × Balb/C) hybrids by lymph node cells, spleen cells and a thymus-bone marrow mixture from C3H mice during the development of casein-induced amyloidosis. The capacity of the thymus-bone marrow mixture to induce GVH-reactions was totally abolished already after ten injections of casein, when the animals not yet are amyloidotic. This reduction in GVH reaction could be restored by thymus cells from untreated donors but not by normal bone marrow cells. After 30 injections of casein, no GVH-activity could be detected in the spleen cells and after 40 injections the GVH-activity of the lymph node cells was only half the normal value. Only the bone marrow cells were unaffected by the casein treatment. Using quantitative comparison of GVH-activity of normal thymocytes and thymocytes from donor mice treated with casein alone or in combination with cortisol parallel line assay showed that the decrease in the capability of thymus cells from casein treated donors to induce GVH reaction was due to a 85 per cent reduction in the number of cortisol resistant thymocytes. The results are discussed in the light of earlier observations on defects in the cellular immune apparatus of mice undergoing casein-induced amyloidosis.

It is well-established that the development of casein-induced amyloidosis in mice and guinea pigs is closely associated with an impairment of the cellular immune apparatus (4 14 15) Thus, the development of tolerance to casein at the cellular level in amyloidotic guinea pigs (4) and a decreased ability to reject allo-skin grafts in amyloidotic mice (14) indicates a severe disturbance in the cellular immune apparatus during the course of amyloidosis. In agreement with these observations we have recently shown that the spleen cells from amyloidotic mice have a decreased capacity to induce a GVH reaction if injected into F -hybrids (9) and have a very low percentage of thetabearing lymphocytes compared to that of untreated mice (10)

In the present study we have examined

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the ability of thymus and bone marrow cells —*alone* and *in mixtures*—spleen and lymph node cells from casein treated mice to induce a GVH reaction in F_1 hybrids. Since the GVH active cells of the thymus are known to be cortisol resistant (2, 3) the effect of casein treatment on these cells was also investigated by cortisol induced decortication of the thymi from casein treated donors. By means of parallel line assay the GVH activity of normal thymocytes was compared to the activity of casein treated and casein-cortisol treated thymocytes.

MATERIAL AND METHODS

Mice Donors were inbred C3H mice of the same age and sex. Recipients were 2–3 month old (C3H × St/A)F_1 and (C3H × Balb/C)F_1 (C3H = H 2^k St/A = H 2^k; Balb/C = H 2^d)

Casein treatment Groups of C3H mice received daily subcutaneous injections of 0.5 ml of a 5 per cent solution of sodium-caseinate for up to 40 days

Cortisol treatment Cortisol-induced decortication of the thymus was achieved by an intraperitoneally injection of 2.5 mg cortisol (LEO) two days prior to sacrifice.

Preparation of cell suspensions All donor mice were about 2 months old when sacrificed Single cell suspensions were made from thymus, spleen, bone marrow and axillary lymph nodes (regional to the sites of casein injections) The cells were washed twice and adjusted in Hanks' Solution (HS) as described below *GVH Assay* A modification of the popliteal lymph node assay in rats described by *Ford et al.* (8) and applied to murine system by *Hardt & Claësson* (9) was used. In the first part of the experiments, the GVH-activity of 5 × 10^6 thymus, spleen, lymph node, bone-marrow cells, and a mixture of 2.5 × 10^6 thymocytes and 2.5 × 10^6 bone-marrow cells was examined. The GVH reaction was calculated at day seven as the ratio between the cell number of the individual popliteal lymph nodes draining the foot pads injected with allogenic cells and the mean cell number of the contra-lateral lymph nodes. This ratio will be referred to as the lymph node index. In the second part of the experiment, the GVH-activity of cortisol resistant thymocytes (CRT) was compared to the activity of CRT from casein treated donors or to that of normal thymocytes by the use of dose-response analysis. The total number of thymocytes in normal mice as well

TABLE 1 *GVH-reactivities of C3H Cells. Popliteal Lymph Node Indices in (C3H × St/A)F_1 Recipients Injected with Lymphoid Cells from Normal C3H Donors and C3H Donors Pretreated with Daily Injections of Casein for Varying Periods of Time The Recipients Received 5 × 10^6 Donor Cells into the Left Foot Pad and Equal Amounts of F Cells into the Right Foot Pad. Each group Represents 7 to 9 Recipients*

Sources of donor cells	Days of casein treatment of donor mice			
	0	10	30	40
2.5 × 10^6 C3H thymocytes + 2.5 × 10^6 C3H BM-cells	2.2 ± 0.3*	1.1 ± 0.2 2.4 ± 0.3† 1.2 ± 0.3‡		0.9 ± 0.2 2.4 ± 0.3†
2.5 × 10^6 C3H thymocytes + 2.5 × 10^6 F BM-cells	1.1 ± 0.1			
2.5 × 10^6 F thymocytes + 2.5 × 10^6 C3H BM-cells	1.2 ± 0.2			
5 × 10^6 C3H thymus cells	1.9 ± 0.2			
5 × 10^6 C3H BM-cells	1.7 ± 0.3			
5 × 10^6 C3H spleen cells	2.3 ± 0.5	2.3 ± 0.6	1.1 ± 0.2	
5 × 10^6 lymph node cells	3.7 ± 0.3	4.3 ± 0.4	4.0 ± 0.8	2.3 ± 0.3

* SEM.
† 2.5 × 10^6 C3H thymocytes from an untreated donor in the thymus-bone marrow mixture.
‡ 2.5 × 10^6 bone marrow cells from an untreated donor in the thymus-bone marrow mixture.

as in groups of mice treated with cortisol and casein-cortisol was determined. All inocula were adjusted to the same cell number (7.5×10^6) by the addition of F_1-hybrid thymocytes. The histological examinations were performed as described previously (5)

RESULTS

The histology of thymus, lymph nodes and spleens of donor C3H mice treated with varying doses of casein revealed the well-known changes described earlier (5 12, 17) After 30 and 40 injections of casein, large amounts of amyloid were seen in the perifollicular position of the spleens. The lymph nodes regional to the sites of injection showed in all groups a pronounced germinal centre activity and an enlargement by several times no amyloid was found. The thymus remained histologically normal in the casein treated groups.

Table 1 shows the GVH-activities of the various lymphoid cell cathegories examined. No GVH-activity was found after injection of either 2.5×10^6 normal C3H thymocytes or 2.5×10^6 normal C3H bone marrow cells into $(C3H \times St/A)F_1$ recipients. A mixture of 2.5×10^6 normal C3H thymus cells and 2.5×10^6 normal C3H bone marrow cells, however revealed a significant GVH-reaction ($p<0.01$) indicating synergism between thymocytes and bone marrow cells.

Table 1 further shows the effect on the GVH reaction of cells recovered from casein treated mice. After ten injections of casein, no GVH activity could be detected in the thymus-bone marrow inoculum while the activity of spleen and lymph node cells still remained at normal levels. After 30 injections of casein, only the lymph node cells showed an intact GVH-activity which after 40 injections had decreased to about half of the normal values. The table furthermore shows that the reduced GVH-activity of the thymus-bone marrow combination could be totally restored by 2.5×10^6 thymus cells from untreated donors but not by 2.5×10^6 normal bone marrow cells. In the 40 days group normal thymus cells could still restore the GVH

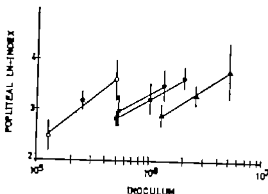


Fig. 1 GVH-reactivity in $(C3H \times Balb/C)F_1$ hybrids of thymocytes from C3H donors. Observed mean popliteal lymph node (LN) indices (± 1 SEM) for various numbers of thymocytes from normal donors (▲—▲) donors treated 48 h previously with 2.5 mg cortisol (○—○) and donors treated with 10 (●—●) and 30 (■—■) injections of casein, respectively followed by treatment with 2.5 mg cortisol 48 h prior to sacrifice.
Each group represents 7 recipients.

activity of thymus-bone marrow mixture indicating normal action of the bone-marrow cells at this time.

The very early decrease in the GVH activity of thymus cells from casein treated donors was studied in greater detail in the second part of the experiment. Figure 1 shows the results from these experiments where the GVH-activity of thymocytes from normal mice was compared with the activity of thymocytes recovered from either cortisol treated mice or casein treated mice injected with cortisol two days before sacrifice.

It appears from the linear regression curves that cortisol treatment alone results in a ten times increase in the GVH-activity of the thymocytes injected while casein-cortisol treatment resulted only in a three times increase. In order to perform a quantitative comparison between the GVH activity of thymocytes from normal and treated mice, the total number of lymphocytes in the thymus from the three groups was determined (Table 2) It is seen that cortisol alone reduces the number of thymocytes to about 10 per cent of the normal level while casein-cortisol treatment reduces the number to about 5 per cent. Thus the position of the regression curves

TABLE 2. *Mean Number of Thymocytes Harvested from 45 Days Old C3H Male Mice*

Treatment*	No. of animals	Cells per thymus
None	5	140×10^6
Cortisol	5	13×10^6
Casein-cortisol	5	6×10^6

* for details see text.

in Fig 1 indicates that cortisol sensitive thymocytes are not responsible for the GVH activity of normal thymus cells. The position of the regression line for casein-cortisol mice collated with the results in Table 2 indicates, however that 85 per cent of the cells responsible for the GVH-activity of normal thymocytes have disappeared from the thymus during the casein treatment.

DISCUSSION

The present results support the hypothesis that the development of casein-induced amyloidosis is closely associated with a functional break-down of the cellular immune system, i.e. the thymus dependent part of the immune apparatus (10) In the first part of the experiment it was shown that increasing injections of casein affect the various lymphoid organs in their ability to elicit a GVH reaction in F_1-hybrids in a certain time sequence The *most sensitive organ to be depleted of* GVH activity in the course of casein treatment is the thymus. Then follows the spleen and it is not until after 40 injections of casein that a decrease in the capability of lymph node cells to elicit GVH reactions is seen.

A mixture of murine thymus and bone marrow cells has been shown to interact synergistic in the GVH reaction elicited in irradiated F_1 hybrids (1 11) Our results are in line with these findings since no GVH activity was found after injection of either 2.5×10^6 C3H thymus or bone marrow cells mixed with an equal number of F_1 cells while 5×10^6 mixed C3H thymus and bone marrow cells showed significant activity even at a level higher than 5×10^6 C3H thymus or bone marrow cells. The thymus-bone marrow combination was used in the present experiment in order to study the influence of casein treatment upon each of the organs' capability to induce a GVH reaction. The present results showed that only the thymus was affected by the casein treatment. The finding that the GVH-activity of the bone marrow cells remains unlatered during casein treatment suggests an intact B-lymphocyte function even in heavy amyloidotic mice.

*It has been shown recently that macro*molecules do not penetrate the vasculo-epithelial barrier of the thymic cortex (16) This can explain the unaltered histological appearance *of the thymus even after heavy antigen* administration (13) On the other hand, the very early depletion of GVH-active thymocyte during the course of casein treatment indicates that administration of certain antigens (e.g casein) acts on a functionally very important but quantitatively very sparse subpopulation of cells within the thymus which —as shown in the second part of the experiment—is resistant to cortisol. Several studies have shown that the number of cortisol resistant thymocytes amounts to 5–10 per cent of the total number of thymocytes and that these cells are responsible for nearly *the total* GVH-activity of the organ (2, 3, 6, 18) In the present study we used a parallel line assay combined with a determination of the total *thymocyte number in normal*, cortisol and casein-cortisol treated donor mice. In agreement with the studies mentioned above, a nearly 10 times enrichment of GVH-active thymocytes was found in the cortisol treated mice together with a decrease in the total thymocytes cell number to about 10 per cent of the normal value, suggesting that the large majority of GVH-active thymocytes belongs to the cortisol resistant cell population. *On* the other hand cortisol in combination with casein pre treatment only raised the GVH activity of the thymocytes by about 3 times, *while it reduced the number of thymocytes* to about 5 per cent of the normal value. This indicates that casein treatment reduces the

number of cortisol resistant thymocytes to about 85 per cent of the normal value. The decreased GVH-activity after 10 injections of casein may refelect a late stress effect caused by the casein injections and mediated by endogenous cortisol production parallel to the late effect of a single injection of cortisol (3). Another possibility could be that casein—and perhaps antigen administration in general—blindfolds thymocytes making them incapable to initiate a GVH-reaction. If so, the present results may indicate that an antigen competition within the thymus gland interfers with the maturation and differentiation processes of thymocytes. The idea of a centrally acting antigen competition seems an attractive supplement to the generally accepted view on antigen competition (7).

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GVH reactivity of such spleen cells was depressed or absent. In addition, we found that the number of θ positive cells was increased in mice transplanted with allogeneic bone marrow cells and that the lack of GVH reactivity was not due to a specific elimination of clones reactive against host histocompatibility antigens. Finally we present suggestive evidence that the lack of GVH activity was not caused by a development of immunological immature θ positive cells either (= T_1's (8)). Possible reasons for the discrepancy between the development of immunological mature T cells in mice transplanted with syngeneic or allogeneic bone marrow cells will be discussed.

MATERIALS AND METHODS

Animals Inbred mice of the following strains were used in this study: A/Sn (H-2^a), C57Bl/6 (H-2^b), Balb/c (H-2^d) and CBA/H (H-2^k). These mice were kept under a strict brother sister breeding scheme (kindly performed by Mr *E. Christensen*). AKR/J (H-2^k) mice were obtained from the animal farm of the Statens Seruminstitut. F_1 hybrid mice were produced by mating three female mice with one male mouse. The following F_1 hybrids were used: CBA × Balb/c, CBA × C57Bl/6, and Balb/c × C57Bl/6.

Irradiation Mice were irradiated with 900 R in a 16 chamber plexiglass box on a General Electric, Maximar 250-III apparatus. The irradiation factors were: 215 kV, 16 mA, focal distance 75 cm, 1 mm Cu and 1 mm Al filters, dose rate 16 R per minute. A Philips integrating dosimeter was used for dose measurements. The irradiation was kindly performed by Mr *Harry Andersen*, Inst. for Exp. Immunology, University of Copenhagen.

Preparation of cell suspensions Spleen cell suspensions were prepared from the spleens of experimental mice by gently pressing the organs through a stainless steel mesh into 10 per cent foetal calf serum/F13 Eagles minimum essential medium (FCS-MEM). Thereafter cell clumps were allowed to settle for about 10 minutes and 4/5th of the supernatant harvested. These cells were washed twice in FCS-MEM and adjusted to the cell concentration needed for the experiments. Bone marrow cells were harvested from the femurs and tibias and lymph node cells from the popliteal, inguinal, and axillary lymph nodes of 8–12 weeks old mice. Thymus cells were from the thymi of 4–6 weeks old mice. All cell suspensions were counted for number of viable cells using the trypan blue-exclusion test (9).

Cell transfer About 2 hours after irradiation, mice were injected with 2–10 × 10^6 syngeneic or allogeneic bone marrow cells intravenously (i.v.). Fifteen minutes before injection, all mice received 100 I.E. of heparin intraperitoneally (i.p.).

Graft-versus-host (GVH) assay Cell suspensions to be tested for GVH activity were injected into 0–3 days old F_1 hybrid mice in a volume of 50 μl (i.p.). The ages of F_1 hybrid mice in a given experiment did not vary by more than one day and there was at least five experimental and three control hybrids in each group. Control mice were injected with FCS-MEM only and their tails were cut for later identification. The spleen enlargement was measured 9 days after injection. Spleen and body weights were measured and the spleen index calculated according to *Simonsen* (10). The results will be given as spleen indices (SI) and as $\log_e SI \pm SE$ (standard error of the mean).

Since the GVH assay in this study was used as a measure of T cell activity we studied the effect of 1) anti-θ antiserum plus complement treatment and 2) passage through anti-Ig coated columns on the GVH activity of spleen cells. As found by others, the former treatment abolished the GVH activity (1), whereas the latter treatment increased the GVH activity parallel to the increase of θ positive cells (14).

Preparation and use of anti-θ antiserum Anti-θ antiserum was raised in female AKR mice injected i.p. and subcutaneously (s.c.) with C3H or CBA thymocytes. Six to eight injections were given weekly (9). Anti-θ (C3H) antiserum was used mostly in these experiments since CBA mice (or their F_1 hybrids) were under test. Lymphoid cells were treated with anti-θ antiserum (about 10^7 cells per 0.1 ml of serum) for 1 hour at 4° C followed by centrifugation and further incubation with fresh, agarose adsorbed (3) guinea pig serum diluted 1:4 in FCS-MEM for ¾ hour at 37° C. The number of live cells was determined by trypan blue (9).

EA and EAC' RFC assays. EA (sheep erythrocytes (SRBC)-anti-SRBC antibody complexes) and EAC' (SRBC-anti-SRBC-complement complexes) rosette forming cells (RFC.s) were determined in order to evaluate the number of B cells in the lymphoid cell suspensions under study (14–16). The antibodies used to form EA complexes were mouse 7S anti-SRBC antibodies. To 10 ml of 2 per cent SRBC was added 50 μl antiserum diluted 1:5 in PBS (Phosphate buffered saline, 1/15M, pH = 7.4). This amount of antiserum was just sub-agglutinating. The mixture was incubated for 30 minutes at 37° C and then washed three times in PBS and finally resuspended in 10 ml FCS-MEM. 0.1 ml of lymphoid cells (1–3 × 10^7/ml) was mixed with 0.1 ml of 2 per cent EA.s, incubated for 1 hour at room temperature and then centrifuged slowly for 10 minutes. The pellet was resuspended carefully in 3.0 ml of FCS-MEM using a pasteur pipette

TABLE 1 *Fractionation Characteristics of Anti-Ig Column Passed Spleen and Bone Marrow Cells**

Cell source	% Θ positive cells	% EA RFC†	% reduction	% EAC'-RFC†	% reduction
Spleen C‡	31 7 (19–45)	22.2 (8–35)	–	28.8 (15–35)	–
" P	86.9 (80–96)	1.8 (0–2)	91.9	1.3 (0–4)	93.5
Bone Marrow C	2.0 (0–3)	6.4 (4–11)	–	10.7 (7–18)	–
" P	2.0 (0–3)	0.8 (0–2)	87.8	5 1 (1–15)	47.2

* Results given in this table are means of 8 individual experiments. Figures in brackets range of the results.

‡ C unpassed control cells P cells passed through an anti-Ig coated column.

† RFC rosette forming cells against EA and EAC' complexes see Materials and Methods.

and lymphoid cells having three or more EA:s adherent to their surface were counted. The results were expressed as per cent EA RFC per total number of lymphoid cells in the suspension.

The antibody used to form EAC' complexes were rabbit 19S anti-SRBC antibodies. These EA:s were prepared as described above. After washing of the EA:s, they were added to 2 ml of fresh mouse serum diluted 1:2 in BSS (balanced salt solution, pH = 6.9) This mixture was incubated for 30 minutes at 37 C. The EAC':s were then washed three times in PBS and resuspended to give a 2 per cent suspension in FCS-MEM. The RFC assay was performed as the assays of EA complexes except that lymphoid cells and EAC':s were centrifuged directly without preceding incubation.

Preparation of columns for cell fractionation Rabbit anti-mouse gamma globulin (Ig) antiserum was obtained by immunizing rabbits with Ig in complete Freund's adjuvant (CFA) Ig was prepared from normal mouse serum by ammonium sulphate precipitation and subsequent fractionation on Sephadex G-200. Glass beads (500 µl in diameter) were first coated with Ig and then with excess rabbit anti-Ig antiserum as described previously (9 14 15) Cell fractionation was carried out at 4 C and the characterization of passed and retained cells has been discussed (9 14 15) In the present studies, lymphoid cell suspensions passed through anti-Ig coated columns were tested before and after passage for anti-Θ positive cells, EA and EAC' RFC:s. The fractionation characteristics of spleen and bone marrow cells used in this study can be seen in Table 1

General design f the experiments 30–45 parental or F hybrid mice were irradiated with 900 R and then divided into groups of at least 6 mice. These different groups received 2–10 × 10⁶ syngeneic or allogeneic bone marrow cells i.v and, at different times after transplantation, the spleens of mice in each group were tested parallel with the spleens of normal mice for their content of Θ positive cells and GVH reactive cells. In some experiments the transplanted bone marrow cells were treated with anti-Θ antiserum plus complement or passed through anti-Ig coated columns before transplantation.

RESULTS

Development of Θ Positive Cells in Mice Transplanted with Syngeneic or Allogeneic Bone Marrow Cells

The development of Θ positive cells after transplantation of bone marrow cells in syngeneic or allogeneic mice can be seen in Table 2 The number of Θ positive cells (T cells) increases with time after transplantation and close to normal levels of T cells are reached 3–4 weeks after bone marrow injection. However a quantitative comparison of the number of Θ positive cells in the spleens of mice transplanted with normal syngeneic or allogeneic bone marrow cells showed that the latter type of mice had significantly greater numbers of Θ positive cells than the former type of mice during the first 3 weeks after transplantation (similar numbers of viable lymphocytes were harvested per animal in the different groups) Anti-Θ antiserum plus complement treatment of the bone marrow cells before allogeneic transplantation showed a similar development in numbers of Θ positive cells compared to normal untreated bone marrow cells. This kind of approach would indicate that the Θ positive cells tested in the bone marrow transplanted mice do not originate from the few

TABLE 2. *Summary of the Development of Θ Positive Cells in the Spleens of Bone Marrow Transplanted Mice*

Transplantation barrier‡	% Θ positive cells per spleen					
	Days after transplantation					
	7–8	10	12–13	15–17	19–21	22–24
Syngeneic BM:N	4.3	6.7	8.4	10.6	15.0	21 1
Allogeneic BM:N	14.0	16.2	20.0	22.9	–	24.5
Allogeneic BM:anti-Θ	12.0	22.0	–	28.2	–	24.9
Allogeneic BM IgP	5.5	8.8	–	21.9	–	–

Results given in this table are means of 5 individual experiments.
‡ Bone marrow cells transplanted to 900 R irradiated mice N = normal, anti-Θ = bone marrow cells treated with anti-Θ antiserum plus complement before transplantation, IgP = anti-Ig column passed bone marrow cells.

Θ positive cells present in normal bone marrow (Table 1)

However if it was attempted to increase the relative proportion of T cells in the bone marrow cell population by passage through anti-Ig coated columns, such passed cells, transplanted into allogeneic mice, showed a development of Θ positive cells in the spleens at a schedule similar to normal bone marrow transplanted into syngeneic mice during the first two weeks after transplantation, i.e. a depressed development of Θ positive cells compared to normal allogeneic bone marrow transplanted mice.

Thus, the amount of Θ positive cells increases more quickly in mice receiving allogeneic bone marrow than in mice receiving syngeneic bone marrow This picture was not changed by removing Θ positive cells from the bone marrow whereas filtration of the bone marrow cells through anti-Ig coated columns caused a slower development of Θ positive cells following allogeneic transplantation. It seems that the confrontation with

TABLE 3. *Summary of the GVH Activity of Spleen Cells from Mice Transplanted with Syngeneic Bone Marrow Cells**

Gr. no.	Cells injected‡	Days after transplantation	% Θ pos. cells	SI†	GVH in CBA × Balb/c mice† log $_e$SI ± SE
1	10^7 N-CBA-Spl.	–	32.6	3 16	0.500±0.022
2	3×10^6	–		1.66	0.220±0.017
3	10^7 Diff. BM	10	6.7	1.23	0.090±0.039
4	3×10^6			1.09	0.037±0 013
5	10^7	17	10.6	1 46	0 166±0.035
6	3×10^6			1.01	0.005±0.017
7	10^7	21	15.0	1.85	0.268±0.023
8	3×10^6			1 43	0.155±0 023
9	10^7	24	21 1	2.30	0.362±0.016
10	3×10^6			1 76	0.245±0.015

*Results are means as presented in Table 2.
‡ 900 R irradiated CBR mice received 5×10^6 CBA bone marrow cells. N-CBA-Spl. = normal CBA spleen cells; Diff. BM = CBA bone marrow cells differentiating in 900 R irradiated CBA mice.
† SI = spleen index SE = standard error of the mean (see also Materials and Methods)

TABLE 4 *Allogeneic Bone Marrow Transplantation of Normal and Anti-θ Antiserum Plus Complement Treated Bone Marrow Cells**

Gr no.	Cells injected§	% θ pos. cells	GVH in CBA × Balb/c SI†	GVH in CBA × Balb/c log SI ± SE	GVH in CBA × C57Bl/6 SI	GVH in CBA × C57Bl/6 log SI ± SE
1	10^7 Gr I Spleen	24.3	1.31	0.118±0.024	–	–
2	Gr II	17.9	1.11	0.044±0.009	–	–
3	Gr III	16.5	1.16	0.065±0.012	–	–
4	10^7 Gr I Spleen	24.3	–	–	1.30	0.115±0.012
5	Gr II	17.9	–	–	1.22	0.085±0.022
6	Gr III	16.5	–	–	1.18	0.073±0.017

* Cells tested day 15 after transplantation.
§ Gr I = 900 R CBA × Balb/c mice inj. with 10^7 A/Sn bone marrow cells. Gr II = 900 R CBA × Balb/c mice inj. with 10^7 CBA bone marrow cells treated with complement. Gr III = 900 R CBA × Balb/c mice inj. with 10^7 CBA bone marrow anti-θ antiserum plus complement treated.
† See Table 3.

histo-incompatible tissue supports T cell proliferation. Subsequently we tested the immunological function of the developing T cells in mice transplanted with bone marrow cells of different origin.

T Cell Function in Bone Marrow Transplanted Mice

Spleen cells from mice transplanted with syngeneic or allogeneic bone marrow cells were harvested at different times after transplantation. Subsequently they were tested for GVH reactivity in newborn F_1 hybrid mice. A summary of the development of GVH reactive cells following syngeneic bone marrow transplantation is given in Table 3. An increase in GVH activity was parallelled by an increase in θ positive cells. However when spleen cells from mice transplanted with allogeneic bone marrow cells were tested, no

TABLE 5 *Effect of Anti-Ig Column Passage of Bone Marrow Cells on Their Development into GVH Reactive Cells in Allogeneic Mice**

Gr no.	Cells injected§	% θ pos. cells	GVH in CBA × Balb/ SI†	GVH in CBA × Balb/ log SI ± SE	GVH in Balb/c × C57Bl/6 SI	GVH in Balb/c × C57Bl/6 log SI ± SE
1	*10 N Balb/c-Spl.*	*31.8*	*2.48*	*0.393±0.010*	–	–
2	3×10^6		2.14	0.331±0.065	–	–
3	10^7 Gr I-Spl.	17.8	1.21	0.084±0.017	–	–
4	3×10^6		1.32	0.119±0.017	–	–
5	10^7 Gr II Spl.	8.1	1.00	0.000±0.022	–	–
6	*3×10^6*		0.99	−0.004±0.024	–	–
7	10^7 N Balb/c-Spl.	31.8	–	–	2.31	0.363±0.021
8	10^7 Gr I-Spl.	17.8	–	–	1.17	0.067±0.022
9	10^7 Gr II-Spl.	8.1	–	–	1.12	0.048±0.026

Cells tested day 22 after transplantation.
§ N Balb/c-Spl. = normal Balb/c spleen cells. Gr I = 900 R CBA × Balb/c mice injected with 4×10^6 Balb/c unpassed bone marrow cells; Gr II = 900 R CBA × Balb/c mice injected with 4×10^6 Balb/c bone marrow cells which have been passed through an anti-Ig coated column.
† See Table 3.

TABLE 6. *Effect of Anti-Ig Column Passage of Bone Marrow Cells on Their Development into GVH Reactive Cells in Allogeneic Mic*

Gr no.	Cells injected‡	% θ pos. cells	GVH in CBA × Balb/c		GVH in CBA × C57Bl/6	
			SI†	log SI ± SE	SI	log SI ± SE
1	2×10^7 N-CBA-Spl.	31 7	4 65	0.667±0.014	–	–
2	4×10^6		1.82	0.260±0.016	–	–
3	2×10^7 Gr I-Spl.	0.0	0.94	–0 027±0.017	–	–
4	4×10^6		0.83	–0.081±0.031	–	–
5	2×10^7 Gr II-Spl.	6.8	1.31	0.117±0.026	–	–
6	4×10^6		1 16	0.065±0.019	–	–
7	2×10^7 Gr III-Spl.	27.2	1.06	0 025±0.019	–	–
8	4×10^6		1 15	0.061±0.013	–	–
9	2×10^7 N-CBA-Spl.	31 7	–	–	4 18	0.621±0.024
10	Gr I-Spl.	0.0	–	–	1.05	0.071±0.035
11	Gr II-Spl.	6.8	–	–	1.33	0.124±0.018
12	Gr III-Spl.	27.2	–	–	1.32	0.121±0.021

Cells tested day 17 after transplantation.
‡ N-CBA-Spl. = normal CBA spleen cells G I = 900 R CBA × Balb/c mice inj with CBA × Balb/c bone marrow Gr II = 900 R CBA × Balb/c mice inj. with CBA bone marrow unpassed. Gr III = 900 R CBA × Balb/c mice inj. with CBA bone marrow passed, through an anti-Ig coated column.
† See Table 3

significant GVH activity would be found during the test-period (12–24 days) see Tables 4–6. From these Tables it can be seen also that the developed θ positive cells were not made *specifically* tolerant against host histocompatibility antigens, since they did not respond against third-party hybrids either

Thereafter the effect of anti-θ antiserum plus complement treatment and passage through anti-Ig coated columns on the development of immunological functionable θ positive cells were tested. From Table 4 it can be seen that anti-θ antiserum plus complement treatment did not alter the development of θ positive cells immunologically inert to transplantation antigens. Neither did passage through anti-Ig coated columns make any difference in this respect (Tables 5 and 6)

The following parameters were changed in order to detect small numbers of GVH reactive cells 1) an increase in the number of cells injected (up to 2×10^7 spleen cells per recipient baby mouse (Table 6)) and 2) passage of spleen cells from mice transplanted with allogeneic bone marrow cells through anti Ig coated columns, which increased the number of θ positive cells from 10–15 per cent up to 50–60 per cent θ positive cells.

TABLE 7 *Synergis Among Spleen and Lymph Node Cells in the GVH Response*

Gr no.	Cells injected	% θ pos. cells	GVH in CBA × Balb/c mice	
			SI†	log SI ± SE
1	2×10^6 CBA spleen	29.3	1.60	0.204±0.032
2	2×10^6 CBA LN	61.5	1.39	0.143±0.021
3	2×10^6 CBA spleen + 2×10^6	–	2.30	0.362±0.010
4	5×10^6 CBA thymus	97.8	1.21	0.071±0.021
5	5×10^6 + 2×10^6 CBA LN	–	1.87	0.272±0.029

† See Table 3.

TABLE 8. *Lack of Synergism Among Spleen Cells from Mice Transplanted with Allogeneic Bone Marrow and Lymph Node Cells from Normal Mice in the GVH Response**

Gr no.	Balb/c cells inj.§		% θ pos. cells	GVH in			
				CBA × Balb/c		Balb/c × C57Bl/6	
	Spleen	Lymph node		SI†	log SI ± SE	SI	log SI ± SE
1	5×10^6	–	33.7	1.83	0.263±0.017	1.61	0.207±0.011
2	–	2×10^6	68.0	1.19	0.076±0.031	1.08	0.034±0.027
3	5×10^6	2×10^6	–	2.47	0.393±0.023	2.51	0.400±0.021
4	10^7 Gr I:a	–	24.2	1.82	0.260±0.024	1.93	0.286±0.026
5		2×10^6	–	1.94	0.294±0.017	2.31	0.364±0.026
6	10 Gr II:a	–	28.7	0.91	−0.041±0.019	1.03	0.013±0.032
7	" "	2×10^6	–	0.96	−0.018±0.033	0.81	−0.091±0.026

* Cells tested day 22 after transplantation.
§ Gr I:a = spleen cells from 900 R Balb/c mice transplanted with 2×10^6 Balb/c bone marrow cells. Gr II:a = spleen cells from 900 R CBA × Balb/c mice transplanted with 2×10^6 Balb/c bone marrow cells.
† See Table 3.

Such manoeuvres were, however unsuccessful.

Finally we tested the possibility that the spleens of mice transplanted with allogeneic bone marrow cells contained θ positive cells of the T_1 category i.e. immunologically immature T cells like those mostly found in the thymus (8). These T_1 cells have been shown to settle in the spleen and their reactivity may be tested using a collaborative GVH assay where spleen and lymph node cells were mixed in such numbers that neither of the two cell populations gave a GVH reaction by themselves (2, 8). That this was true of normal spleen and lymph node cells in the present test system was confirmed by the experiment presented in Table 7. Spleen and thymus cells were able to collaborate with lymph node cells to give a GVH reaction. Accordingly we tested the ability of spleen cells from mice transplanted with syngeneic or allogeneic bone marrow to give a collaborative specific GVH response with lymph node cells syngeneic to the transplanted bone marrow cells. From Table 8 it is obvious that spleens from mice transplanted with allogeneic bone marrow cells gave neither a specific nor a third-party collaborative GVH response. Thus, the failure of θ positive cells in the spleens of mice transplanted with allogeneic bone marrow cells to respond against histocompatibility antigens seems not to be due to a T_1 immunological status of such cells. On the other hand θ positive cells in the spleens of mice transplanted with syngeneic bone marrow gave a GVH response, but a rather poor collaborative GVH response (Table 8).

DISCUSSION

Lethally irradiated mice transplanted with syngeneic bone marrow cells are fully restored haematopoietically and regain their immunocompetence 4–6 weeks after transplantation (4–7, 11–13). In contrast, if reconstituted with allogeneic bone marrow cells, such mice often suffered from secondary disease and their immunocompetence did not regenerate normally mainly due to a suppressed T cell function (6, 12). In the present communication we have analysed some reasons for the anomaly e.g. 1) is the number of T cells to develop in the spleens of mice transplanted with allogeneic bone marrow lower than the number of T cells in their syngeneic counterparts? 2) is the abnormal development of T cell function due to a production of inhibiting substances produced by mature T cells in the allogeneic bone marrow upon tolerance

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SPECIFICITY OF RHEUMATOID FACTORS CROSS-REACTING WITH HUMAN AND RABBIT IgG

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The reaction of rheumatoid factors with rabbit IgG sensitized erythrocytes was inhibited by Fc fragments and by a mixture of Facb and pFc' fragments of rabbit IgG but not by either of the latter two fragments alone. The reaction was also inhibited by heat aggregated human IgG1 and IgG2 myeloma proteins, depending on the amount of aggregates present, but irrespective of their Gm types. No inhibition was seen with IgG3 or IgG4 proteins. Immunosorbent studies showed that the antigen of human IgG involved in these reactions is probably closely related to the γ1 2-4 antigen, which is a major human IgG antigen interacting with rheumatoid factors. Quantitative haemagglutination inhibition studies in an Auto-Analyzer® showed that native human IgG very weakly inhibited the reaction of rheumatoid sera with rabbit IgG. Isolated aggregated human IgG inhibited far better, and gave an inhibition curve indicating the formation in aggregated human IgG of new antigens very similar to antigens of native rabbit IgG. Thus, in rheumatoid arthritis there may be a humoral immune response also against antigens present in aggregated but not in native human IgG, giving the cross-reaction with native rabbit IgG.

Rheumatoid factors are human antibodies to various antigens of the Fc part of IgG. Although probably directed primarily against human IgG, they may also react with IgG from a range of mammalian species (11). The reactivity with rabbit IgG forms the basis for the Waaler Rose test, which is used diagnostically for the detection of rheumatoid factors (22, 25).

Although the reaction is known to take place with the Fc region of rabbit IgG, recent work showed that neither Facb nor pFc' fragments, representing homology regions Cγ2 and Cγ3 of the Fc region, respectively, alone could neutralize the rheumatoid factors reacting with intact rabbit IgG (24).

Absorption studies using aggregates or antigen-antibody precipitates containing human IgG indicate that the reactivity of rheumatoid factors with rabbit IgG represents a cross-reaction of rabbit IgG with human IgG (1, 2, 9). Apart from that, however, very little is known concerning the human IgG antigens towards which the rheumatoid factors reacting with rabbit IgG are directed.

The purpose of the present investigation was to further characterize the IgG antigens involved in the reaction of rheumatoid factors with rabbit IgG.

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MATERIALS AND METHODS

Antisera

Sera from 14 randomly selected patients with seropositive rheumatoid arthritis were obtained from the Oslo Sanitetsforening Rheumatism Hospital, and were stored at −20° C until use. Before use they were inactivated at 56° C for 30 min. As these sera all showed the same reaction pattern with rabbit IgG, results obtained with a pool of them will be given in this paper. In addition, for the detection of the γ1 2-4 (Ga) and "non a" antigens, the IgM fractions of selected rheumatoid sera obtained by gel filtration on Sephadex G-200 at pH 4.0 were used as previously described (5).

Haemagglutination Tests

For amboceptor production, rabbits were immunized intravenously with human OR_1R_1 erythrocytes. For sensitization, a 10 per cent suspension of washed erythrocytes was incubated at 37° C for 30 min with constant agitation with approx. 1/3 of an agglutinating amount of IgG from a pool of two rabbit antisera. After three washings with PBS (phosphate buffered saline pH 7.4) the sensitized cells were used as a 1 per cent or 8 per cent suspension in PBS for the manual and automated techniques, respectively.

Haemagglutination inhibition tests with rabbit IgG sensitized erythrocytes were performed at room temperature on glass plates with shallow curved troughs. In each trough, one drop of antiserum (diluted in PBS to give a 2–3+ reaction, usually corresponding to approx. 4 agglutinating units) was allowed to react for 5 min with one drop of the solution to be tested for inhibition, after which one drop of 1 per cent sensitized erythrocytes was added. The agglutinations were read after 15 min of relatively vigorous agitation on a horizontal shaking machine.

Gm typing and detection of the γ1 2-4 and "non a" antigens were carried out in tubes by a routine procedure (5).

Quantitative haemagglutination inhibition experiments were performed with a Technicon AutoAnalyzer® as described elsewhere (8). The percentages of inhibition obtained with various concentrations of an inhibitor were plotted against the concentrations on graph paper to give an inhibition curve for each inhibitor (8).

IgG Preparations

Pooled human IgG was obtained as a lyophilized material or a 16.5 per cent solution from Kabi AB, Sweden. Since no high molecular weight aggregates were demonstrable in the latter preparation by gel filtration or sucrose gradient ultracentrifugation, it was used as native IgG in this study. IgG from normal rabbit sera and from normal human Gm(a+f−b−g+) and Gm(a−f+b+g−) sera, respectively, was isolated by column ion exchange chromatography on DEAE-cellulose (DE-52, Whatman, England) in 0.015 M phosphate buffer pH 7.6.

Human IgG myeloma proteins were isolated from the sera of patients with multiple myeloma by starch block electrophoresis or by ion exchange chromatography on DEAE-cellulose or QAE-Sephadex and, if necessary, rechromatographed and gel filtrated on Sephadex G-200 (8).

$F(ab')_2$, Fab, Fc, and pFc′ fragments of pooled human IgG were prepared as previously described (8).

The *preparations of human IgG enzymatic fragments* and myeloma proteins were tested for purity in sensitive haemagglutination inhibition tests using defined antigenic marker systems (19).

$F(ab')_2$, Fab, Fc, and pFc′ fragments of pooled rabbit IgG were prepared as the corresponding human fragments. Rabbit Fab/c was obtained by papain digestion followed by Sephadex G-150 gel filtration (13). Facb fragments were obtained by plasmin digestion (3, 24) followed by Sephadex G-100 gel filtration in dissociating media. The rabbit IgG fragments were tested for purity by double immunodiffusion in agarose using sheep antisera specific for rabbit whole IgG and Fc. Their identification was also supported by molecular weight determinations by gel filtration on calibrated Sephadex G-100 and G-150 columns and by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (13, 14).

Gammaglobulins of hen, mouse, guinea pig, hamster, rat, sheep, ox and horse sera were isolated by two successive ammonium sulphate precipitations at half saturation, after which the precipitates were dissolved in PBS and dialysed against PBS. By agarose gel electrophoresis they were shown to contain mostly γ-globulin, but were, to a varying extent, contaminated by albumin and small amounts of other serum proteins.

Aggregation of IgG Preparations

IgG, IgG fragments or γ-globulin preparations at 7 to 20 mg/ml in PBS were heat aggregated at 63° C for 12 minutes. The amount of aggregated material formed by heat denaturation was measured after preparative ultracentrifugation on a sucrose gradient as previously described (8).

Gel Filtration

Plasmin digested rabbit IgG was gel filtrated on Sephadex G-100 in either 6M urea in 0.2 M acetate buffer pH 4.5, 5 M guanidine in 1 M acetic acid or 0.01 M tris buffer pH 7.6 in 0.15 N NaCl.

Rheumatoid sera were gel filtrated on Sephadex G-200 using a 0.1 M acetate buffer pH 4.0 in

0.2 N NaCl as previously described (5). In some instances, the IgM fraction was refiltrated in order to minimize the IgG contamination.

Isolation of the aggregated human IgG formed by heating was performed by gel filtration on Sepharose 6B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in PBS containing 0.1 per cent bovine serum albumin (BSA) as previously described (4, 8). To obtain aggregates of different molecular sizes, heat denatured IgG was subjected to gel filtration on Sepharose 2B (which, according to the manufacturer, excludes proteins of molecular weight 40×10^6 and above) in a 1.0 cm $\times$ 88 cm column eluted at room temperature by upward flow with PBS containing 0.1 per cent BSA.

Immunosorbent Technique

15 mg of IgG from three normal rabbit sera, 88 mg of heat aggregated pooled human IgG, 5 mg of an IgG3 Gm(b) myeloma protein (Dow), 4.5 mg of an IgG3 Gm(g) myeloma protein (Her), 6 mg of an IgG4 myeloma protein (JJo), and 5 mg of human pFc' fragments were coupled to 6-9 g aliquots (wet weight) of Sepharose 2B by means of cyanogen bromide (13) and packed in separate columns. 2 ml of the rheumatoid serum pool, diluted 1/4 in PBS, were recycled through the immunosorbent column overnight at +4° C. After extensive washing with PBS, the bound rheumatoid factors were eluted by 0.1 M acetate buffer pH 4.0 in 0.2 N NaCl at room temperature, the pH was brought up to between 7 and 8 with solid tris salt, and the eluate was concentrated by gentle negative pressure in dialysis tubing. The same procedure was also performed with a corresponding amount of the IgM fraction of rheumatoid serum pool isolated by two Sephadex G-200 gel filtrations at pH 4.0.

RESULTS

Reaction with Native (Untreated) and Aggregated Rabbit IgG

In haemagglutination experiments on glass plates, native pooled rabbit IgG inhibited the reaction of rheumatoid sera with rabbit IgG sensitized red cells at 0.25 to 0.5 mg/ml. After heat aggregation it inhibited slightly better (Table 1). No difference in inhibitory capacity of IgG preparations from six individual rabbits was observed.

In the Auto-Analyzer (Fig. 1) native rabbit IgG gave 50 per cent inhibition at 0.6 mg/ml. After heat aggregation the rabbit IgG

*TABLE 1. The Reaction between the Rheumatoid Serum Pool and Rabbit IgG Inhibited by Rabbit IgG Preparations**

Inhibitor	Lowest concentration of untreated and heat aggregated inhibitor in mg/ml giving full inhibition	
	Untreated	Heat aggregated
Whole IgG	0.25–0.5	0.12
F(ab')$_2$	>4.0	>4.0
Fab	>4.0	>4.0
Fc	0.25	0.12
Fab/c	0.25	0.25
Facb§	>4.0	>4.0
Facb + pFc'†	0.25	0.12
Facb + pFc'‡	0.5	0.5
pFc'	>2.0	>2.0

* Prepared from pooled rabbit IgG.

§ Separated from pFc' by gel filtration in 5 M guanidine/1 M acetic acid or in 6 M urea/0.2 M acetate buffer pH 4.5.

† Plasmin digested IgG subjected to gel filtration in 0.01 M tris buffer pH 7.6 in 0.15 N NaCl.

‡ A mixture of isolated Facb and pFc' fragments, molar ratio 1:2.

reacted somewhat more strongly, producing 50 per cent inhibition at 0.24 mg/ml but the inhibition curves achieved were consistently somewhat less steep than those of native rabbit IgG.

Although a considerable amount of aggregates (18–20 per cent) was formed in rabbit IgG after heating at 63° C, it was never possible to isolate these aggregates by gel filtration. Presumably they precipitated in the column. Attempts to isolate the aggregates by reducing the heat denaturation time down to one minute, which still caused visible aggregation, were also unsuccessful.

Reaction with Enzymatic Fragments of Rabbit IgG

Inhibition was obtained with rabbit Fc and Fab/c fragments, whereas rabbit F(ab')$_2$, Fab and pFc' were all negative (Table 1). Facb fragments of pooled rabbit IgG separated from pFc' by gel filtration in a dissociating medium (guanidine or urea at low pH) did not give any inhibition. However, the same pre-

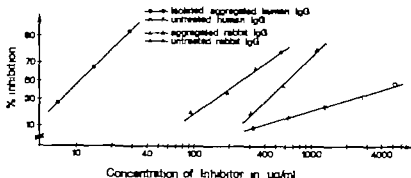


Fig 1 The reaction between the rheumatoid serum pool and rabbit IgG sensitized erythrocytes inhibited by untreated and aggregated human and rabbit pooled IgG.

paration, subjected to gel filtration in a non-dissociating medium (tris buffer pH 7 6) where only one protein peak was obtained, gave a clear inhibition. Inhibition was also seen with a mixture of Facb and pFc fragments, although at a somewhat higher concentration than with Fc fragments. No or only slight increase in the inhibiting capacity was observed with heat aggregated preparations.

Reaction with Native (Untreated) and Aggregated Human IgG

The reaction of the rheumatoid serum pool with rabbit IgG sensitized erythrocytes was inhibited by native pooled human IgG in high concentrations. After heat aggregation the inhibitory capacity was greatly increased (Table 2) No difference in inhibitory capacity of heat aggregated IgG from Gm(a+f-b-g+) and Gm(a-f+b+g-) sera, respectively was found.

When tested in the Auto-Analyzer (Fig 1) native IgG gave a relatively weak inhibition, giving 50 per cent inhibition at 6.5 mg/ml. Aggregated pooled human IgG isolated on Sepharose 6B, reacted far better giving 50 per cent inhibition at only 0 011 mg/ml. Also the slopes of the inhibition curves of native and isolated aggregated human IgG differed considerably that of native IgG being much less steep than that of isolated aggregated IgG

All the anti-rabbit IgG activity was removed (titre reduction from 1024 to less than four) by passing 1 ml of the rheumatoid serum pool through the immunosorbent containing aggregated human IgG

Reaction with Enzymatic Fragments of Human IgG

The reaction of rheumatoid sera with rabbit IgG was clearly inhibited by Fc fragments of pooled human IgG F(ab)₂, Fab and pFc fragments were all negative (Table 2) Heat aggregation produced no change in inhibiting capacity of the fragments.

Relation to Aggregate Size

Sepharose 2B gel filtration of heat aggregated pooled human IgG regularly gave three protein peaks (Fig 2) The first peak (fraction no. 15) which was eluted in the void volume contained the highest molecular weight aggregates. Intermediate size aggregates were eluted in a broad second peak (fractions no. 23 and 26) whereas the third peak (including fraction no. 34) contained the smallest aggregates and the non-aggregated IgG

Six of the fractions collected were studied in the Auto-Analyzer As shown in Fig 3, fraction 34 gave only a weak inhibition and a slowly rising inhibition curve. The material of fraction 26 gave nearly maximal inhibition, that of fractions 23 and 19 inhibited even slightly better whereas fraction 15 with the largest aggregates consistently gave somewhat

TABLE 2. *The Reaction between the Rheumatoid Serum Pool and Rabbit IgG Inhibited by Human IgG Preparations*

Inhibitor	Lowest concentration of untreated and heat aggregated inhibitor in mg/ml giving full inhibition		Per cent aggregates in heat treated preparations*
	Untreated	Heat aggregated	
Pooled IgG	4.0–8.0	0.25	24
Gm(a+f–b–g+) IgG	4.0	0.12–0.25	20
Gm(a–f+b+g–) IgG	4.0	0.12–0.25	18
F(ab')$_2$	>4.0	>4.0	9
Fab	>4.0	>4.0	–‡
Fc	1.0–2.0	1.0–2.0	15
pFc'	>4.0	>4.0	10

All untreated preparations contained less than 5 per cent of aggregates.
‡ Not determined.

weaker inhibition. The slopes of the inhibition curves also gradually increased from the native IgG and smallest aggregates to the highest molecular weight aggregates, fractions 15 and 19 giving maximal steepness. The inhibition curves of the intermediate size aggregates (fractions 23 and 26) giving nearly maximal inhibition, were definitely somewhat less steep than those of the highest molecular weight aggregates (fractions 15 and 19)

Relation to IgG Subclasses (Table 3)

Inhibition of the reaction of rheumatoid sera with rabbit IgG was achieved with heat

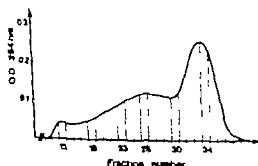


Fig. 2 Sepharose 2B gel filtration of heat aggregated pooled human IgG. Blue dextran (Pharmacia, Uppsala, Sweden) gave an initial peak corresponding to that of fraction 15 and keyhole limpet haemocyanin (Calbiochem AG Lucerne, Switzerland, molecular weight approx. 3,000,000) gave peak corresponding to fraction 30.

aggregated myeloma proteins of subclasses IgG1 and IgG2 irrespective of their Gm types. The degree of inhibition was clearly related to the amount of aggregates formed. No inhibition was observed with aggregated IgG3 or IgG4 myeloma proteins.

Possible Relation to Human Shared Isotypic Antigens

The inhibition of the reaction between rheumatoid sera and rabbit IgG with heat aggregated myeloma proteins of subclasses IgG1 and IgG2 only suggested that the human shared isotypic antigens γ1 2-4 and "non a" which occur together in these two subclasses only (19) might be involved in these reactions.

Individual and pooled rabbit IgG preparations inhibited the reaction of rheumatoid anti-γ1 2-4 antibodies with human IgG coated erythrocytes at concentrations 0.5–2.0 mg/ml. After heat aggregation, the rabbit IgG showed no inhibition at 8 mg/ml. Neither untreated nor heat denatured rabbit IgG inhibited rheumatoid anti-"non a" at 8 mg/ml. None of the untreated or heat denatured gamma globulin preparations from the other animal species inhibited rheumatoid anti-γ1 2-4 or anti "non a" at 4 mg/ml.

Passage of the rheumatoid serum pool through the rabbit IgG immunosorbent removed all the anti rabbit IgG activity

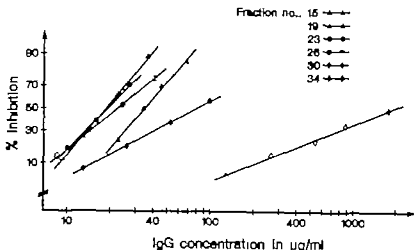


Fig 3 The reaction between the rheumatoid serum pool and rabbit IgG sensitized erythrocytes inhibited by various fractions of Sepharose 2B gel filtration of heat aggregated pooled human IgG (Fig. 2)

while it caused practically no titre reduction against IgG1 Gm(a) or Gm(f) sensitized red cells. All the rheumatoid factor activity against rabbit IgG could be eluted from the immunosorbent column with acetate buffer pH 4 0 In contrast, this eluate contained only a weak activity against human Gm(a) and Gm(f) sensitized cells. The reaction of the eluate with Gm(a) sensitized cells was inhibited by untreated human IgG1 IgG2 and IgG4 proteins but not by IgG3 thus showing a clear γ1 2-4 specificity Similar immunosorbent studies of the twice gel filtrated IgM fraction of the rheumatoid serum pool were also done, and these gave identical results.

The rheumatoid serum pool was also passed through human pFc IgG3 or IgG4 immunosorbents, respectively The serum samples thus absorbed still showed the same specificity in the reaction with rabbit IgG being inhibited by aggregated human IgG1 and IgG2 proteins only

DISCUSSION

Rheumatoid factors are known to react with the Fc part of IgG from a variety of animal species. The reaction with rabbit IgG which is utilized in the Waaler Rose test, has been most thoroughly studied. It is presumed to represent a cross-reaction with human IgG (1 2, 9 10 12 21 26) This concept was supported by the present study where all the reactivity with rabbit IgG could be removed by absorption with aggregated human IgG In addition, several rheumatoid factor specificities against strictly human or primate IgG antigens are known (5 6 7 17 18, 19) The existence of rheumatoid factors reacting primarily with rabbit IgG and other mammalian IgG has also been claimed (15 16, 20, 23 26) However it has hardly been proven that they react exclusively with non-human IgG and not at all with any type of human IgG On the contrary rheumatoid factor preparations reacting with rabbit IgG but unable to agglutinate human IgG sensitized red cells, were shown by haemagglutination inhibition technique to react with heat aggregated human γ-globulin (26)

The reaction of sera with rabbit IgG was inhibited by native human IgG in haemagglutination inhibition tests, although high concentrations of the inhibitor were needed. As expected aggregated human IgG reacted far better Moreover when native and isolated aggregated IgG were tested in the AutoAnalyzer the inhibition curves were clearly unparallel, that of isolated aggregated human

TABLE 3. *The Reaction between Pooled Rheumatoid Sera and Rabbit IgG Inhibited by Various Heat Aggregated IgG Myeloma Proteins*

Inhibitor			aggregates	Lowest concentration in mg/ml giving full inhibition
IgG1	Gm(a)	KNI	<5	4.0
		KOl	<5	2.0
		Oru	5	1.0
		Svo	8	1.0
		Gra	11	1.0
		Bro	62	0 12
		Fay	64	0.03-0.06
	Gm(f)	Sm	<5	>4.0
		Djo	<5	2.0
		Gre	5	1.0
		Ber	16	0.25
		Bol	48	0.12
		Svi	48	0.06
		Tro	82	0.06
IgG2	Gm( )	New	<5	>4.0
		Ler	<5	>4.0
		Edm	40	0.06
	Gm(n-)	Tsch	<5	>4.0
		Smi	94	0.03
IgG3	Gm(b)	Whl	<5	>4.0
		Bra	15	>4 0
		Pro	50	>4 0
		Dow	52	>4.0
	Gm(g)	Ska	8	>4 0
		Vll	14	>4.0
		Yas	22	>4.0
		Har	60	>4.0
IgG4		Ger	<5	>4.0
		Lee	10	>4.0
		Llo	20	>4.0
		Jjo	26	>4.0

IgG being much steeper than that of native human IgG This suggests that the heat aggregation procedure creates or demasks new antigenic determinants in the human IgG giving a better cross-reaction with rabbit IgG This is, to some degree, in contrast to the finding with rheumatoid anti-Gm(a) where new reactive antigenic determinants of either Gm(a-) or Gm(a+) human IgG are probably not formed upon heat aggregation, as indicated by the closely parallel curves obtained with both native and heat aggregated Gm(a-) and Gm(a+) IgG (8)

Untreated rabbit IgG gave an inhibition curve parallel to that of isolated aggregated human IgG suggesting an antigenic similarity between the two preparations. Even though aggregated rabbit IgG reacted somewhat more efficiently than untreated rabbit IgG the inhibition curves obtained with aggregated rabbit IgG were consistently somewhat less steep than those of untreated rabbit IgG suggesting in addition to the, maybe unspecifically increased reactivity of aggregated IgG a change in antigenic specificity away from that responsible for the cross-reaction between untreated rabbit IgG and aggregated human IgG

Sepharose 2B gel filtration experiments showed that the degree of cross-reactivity with rabbit IgG increased with increasing aggregate size of human IgG up to aggregates slightly retarded on the columns and therefore probably of a molecular weight of somewhat less than 40 millions. The highest molecular weight aggregates (molecular weight 40 millions and above) however reacted slightly less efficiently although they gave an inhibition curve parallel to that of the best reacting aggregates. The reduced inhibiting capacity of the largest aggregates may mean that, in aggregates above a certain size, some of the reactive antigenic sites become unavailable for the rheumatoid factor molecules.

The cross-reactivity between human and rabbit IgG was clearly related to subclasses of human IgG Inhibition studies showed reaction with aggregated myeloma proteins of subclasses IgG1 and IgG2 only irrespective of their genetic types. Moreover the reactivity of the IgG1 and IgG2 proteins was clearly related to the amount of aggregates present. Analogously the reaction of rheumatoid anti-Gm antibodies with aggregated IgG myeloma proteins negative for the particular Gm types was also dependent on the amount of aggregates formed (8) In contrast to the latter reactions, however aggregated IgG3 proteins did not in any case inhibit the reaction of rheumatoid sera with rabbit IgG

The reaction pattern with aggregated myeloma proteins suggested that the human

isotypic antigens γ1 2-4 and "non a" to which rheumatoid factors are frequently directed (5) might be involved in the cross-reactions of human IgG with rabbit IgG However the reaction of IgG1 Gm(a) proteins, but not of any IgG3 or IgG4 proteins, all three groups containing only one of the two shared isotypic antigens mentioned, clearly indicated that this could not be the whole explanation. *Moreover the reaction of rheumatoid* anti "non a" with positive human IgG could not be inhibited with rabbit IgG and no evidence of anti-"non a" activity was obtained in the rheumatoid factors reacting with rabbit IgG isolated by immunosorbent technique. On the other hand, rheumatoid anti-γ1 2-4 was clearly inhibited by untreated rabbit IgG although at higher concentrations than positive human IgG (5) In addition, rheumatoid factors reacting with rabbit IgG isolated by immunosorbent technique, gave a clear inhibition with IgG1 IgG2 and IgG4 proteins if tested with human IgG-coated red cells. Absorption experiments with immunosorbent columns containing IgG3 or IgG4 myeloma proteins of human pFc fragments did not reveal rheumatoid factors reacting with rabbit IgG other than those *reacting with human IgG1 and IgG2 proteins.*

Thus rabbit IgG contains a weak γ1 2-4 antigenic activity which is abolished by heat aggregation (possibly reflected also by the changed slope of the aggregated rabbit IgG inhibition curve with the Auto-Analyzer) Moreover the rheumatoid factors reacting with rabbit IgG do show an anti-γ1 2-4 specificity if tested with human IgG-coated red cells, but an anti-γ1 2 specificity if tested with rabbit IgG-coated red cells. Thus, the antigen of aggregated human IgG cross-reacting with rabbit IgG in the Waaler Rose reaction may not be identical with the γ1 2-4 antigen, which is a major human antigen involved in rheumatoid factor reactions, but rather some closely related antigen, possibly dependent also on structures surrounding the γ1 2-4 antigenic site in aggregated IgG1 and IgG2 proteins and absent from IgG4 proteins. The reaction to the γ1 2-4 antigen is also supported by studies in which enzymatic fragments of human IgG were used, which showed the cross-reacting antigen of human IgG to be located on Fc but not on pFc and therefore probably on homology region Cγ2 where the γ1 2-4 antigen is also situated.

Although Fc fragments of rabbit IgG *reacted with rheumatoid anti-rabbit IgG* neither pFc' fragments (representing homology region Cγ3) nor Facb fragments (representing homology region Cγ2) alone showed any inhibiting capacity as also shown by others (24) However inhibition was retained when the plasmin digested IgG was gel filtrated under neutral non-dissociating conditions or when isolated Facb and pFc fragments were mixed. This shows that the lack of activity in either Facb or pFc fragments is not due to loss of some portion of the gamma chain during the plasmin treatment. In fact, there may be two populations of rheumatoid anti-rabbit IgG one reacting with Cγ2 and the other with the Cγ3 region of rabbit IgG Or there may be one antigenic site depending on the co-existence or integrity of both of these homology regions.

Some of the myeloma proteins studied were kindly supplied by Dr *H G Kunkel*. Mrs *Ghislaine Lang* and Mrs *Kari Torbergsen* provided skilled technical assistance.

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BRIEF REPORT

RUBELLAVIRUS-SPECIFIC IgM AND IgA-ANTIBODIES. THE INDIRECT IMMUNOFLUORESCENCE (IF) TECHNIQUE APPLIED TO SERA WITH REDUCED IgG-CONCENTRATION

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It has been demonstrated that rubellavirus IgM and probably also IgA (serum)-antibodies are present only in the acute phase of postnatal rubella infections (3 4) The demonstration of these classes of rubella-specific immunoglobulins has therefore been found to be of diagnostic value, especially in cases where titre increases could not be found employing the HI-method.

The IF-technique should offer good possibilities for determination of heavy-chain-specific viral antibodies (1 4 7 8, 10, 11) Nevertheless several laboratories have experienced difficulties in obtaining reproducible results when this technique has been applied to unfractionated sera collected during infection with rubella- cytomegalo- or Epstein-Barr (EB)-viruses.

As the presence of specific IgG-antibodies can interfere with the determination of specific IgM antibodies by the IF-technique (1 4 11) we have investigated some methods other than ultracentrifugation and column-chromatography for the separation of IgG from the other immunoglobulins Lowering the IgG-content of the serum sample by absorption with DEAE-Sephadex 50 as originally described by *Webb* (15) has been found to be a suitable method for this purpose. This report describes the preliminary results obtained by applying the IF-technique to sera absorbed with DEAE-Sephadex-50 and the comparison of this method with the method where sucrose density gradient centrifugation is applied to the serum samples (3 5 6 7 12)

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Material and Methods

Sera examined. Thirty-two pairs of sera from patients with primary postnatal rubella infections showing titre increases by HI-tests. The sera were obtained during the first four weeks following the rash, and there was on an average 11 7 days between the sampling of the individual serum pairs

Haemagglutination-inhibition (HI)-test employed The sera were examined in the Takatsy-microtitration system, using essentially the technique described by *Halonen et al.* (9)

Determination of IgA IgG and IgM in sera and serum fractions. This was done by rocket immunoelectrophoresis in antibody-containing gels, as described by *Weeke* (14) The heavy-chain-specific antihuman antisera employed were obtained from Dakopatts Ltd., Copenhagen. The immunoglobulin standard used was a pool of human sera calibrated against the WHO reference preparation 67/97

Sucrose density gradient centrifugation. This was done using 0.5 ml of a 1 2 dilution of the serum sample layered on top of a 12 5–37.0 per cent (w/v) sucrose gradient and centrifuged overnight (10^5 *g*, 16–17 h) as described by *Best et al.* (3)

Indirect fluorescent antibody staining 1) *Antigen.* Rabbit cornea cells (SIRC-cells) infected with the Judith strain of rubella virus and maintained in Sella medium were employed. Ordinary microscopic glass slides were each supplied with 8 spots of cells from a suspension of these cells (75×10^3 cells/spot) The slides were fixed in acetone for 20 min at –20 C.) *Conjugates.* Rabbit-antihuman IgG- IgA and IgM-conjugates were obtained from Dakopatts Ltd., Copenhagen, and absorbed with uninfected SIRC-cells before use. 3) *Staining technique employed* *a.* Incubation of slides with serum dilutions or sucrose-gradient fractions for 1 h at 37 C *b* Washing twice in PBS. *c* Conjugate added for 45 min at 37 C. *d* Washing twice in PBS. *e* Application of 20 per cent glycerol in TRIS-buffer (pH 8.4) plus coverslip.

When serum fractions obtained after DEAE Sephadex treatment of the sera (see below) were

investigated by the IF-technique, the slides were preliminarily incubated with fresh rabbit serum (30 min) followed by washing twice in PBS (10 min) and once in distilled water (2 min). This treatment was found to reduce non-specific staining.

Lowering the IgG content of the test sera by treatment with DEAE-Sephadex 50 (Pharmacia Chemicals, Sweden). This was done essentially according to the method described by *Webb* (13). T 3 ml of the Sephadex-gel (in plastic tubes) prepared as described (13) was added 0.5 ml of the test serum, and the tube was shaken manually for 10 min followed by centrifugation at 10^3 g for 10 min. The IgG-containing supernatant was discarded and the gel washed ten times in 0.02 M phosphate-buffer. The buffer was discarded and 0.5 ml of a 16 per cent saline solution was added to the gel which was then shaken for 10 min. The IgM-containing supernatant (eluate) obtained after centrifugation for 10 min (10^3 g) was dialysed overnight against PBS and finally filtered through a membrane filter to remove any remaining Sephadex-particles. The membrane filters, obtained from Sartorius (Göttingen, West Germany) had a pore size of 450 nm and a diameter of 13 mm.

Results

The immunoglobulin content of the serum samples treated with DEAE-Sephadex 50 were investigated. As mentioned above, 0.5 ml of the serum samples was added to 3-ml-volumes of Sephadex gel, but after final dialysation against PBS the IgM-containing eluates from the gel had reached a volume of approx. 1.5 ml. It was found that from about 30 to 50 per cent of the IgM and from about 5 to 10 per cent of the IgG-content present in the original serum samples were recovered in the final dialysed eluates. This means that the ratio between IgG and IgM (in mg per 100 ml) was altered from approximately 10:1 to approximately 2:1. Furthermore it was found that 30–50 per cent of the IgA-content present in the original serum samples were recovered in the final, dialysed eluates.

Particular attention was paid to the specificity of the assay methods used for rubella IgM-antibody determination. The fractions obtained by sucrose gradient centrifugation were all tested for contents of IgG, IgM and IgA by rocket immunoelectrophoresis. Usually IgG and IgA were not present in the first 4 or 5 fractions (0.3 ml each). When the first three fractions showed definite inhibition of rubellavirus haemagglutination, we felt rather sure of scoring the serum as rubella IgM positive.

For several of the test sera concerned, pools were made of fractions Nos 1–3 and of fractions Nos 7–10 obtained after sucrose gradient centrifugation. These pools were dialysed overnight against PBS and concentrated to half the original volume by vacuumdialysis. The pools were subsequently tested for rubella antibodies by indirect IF as described above. The anti-IgM-conjugates used in the present investigation were found only to give fluorescence with the IgM-containing pools which, on the other hand, were not stained by the anti-IgG-conjugate used.

HI (performed on sucrose gradient fractions) and IF (performed with DEAE-Sephadex 50 absorbed serum fractions) were compared as methods for rubella IgM-antibody determination. Thirteen pairs of sera, showing HI-titre increases and obtained from patients with primary postnatal infections, were investigated. The IgG-titres determined by IF (performed on unfractionated sera) closely followed the HI-titres, and all 15 pairs of tested sera also showed IgG-antibody titre-increase by IF. Nine of the serum pairs showed presence of specific IgM-antibodies when tested by sucrose gradient centrifugation, and 11 of the serum pairs when tested by IF. One serum showed presence of IgM-antibodies only when tested by IF and another serum showed IgM-antibodies only when tested by sucrose gradient centrifugation. Two sera were not readable (unspecific agglutination) by the HI test when the first sucrose fractions were tested for specific IgM-antigodies, but these antibodies were demonstrated by IF. The IgM-antibody titres obtained by IF were calculated to be in the range of 6–24.

The presence of virus-specific IgM-antibodies and the presence of virus-specific IgA-antibodies were compared in sera from patients with primary rubella infections. A total of 19 pairs of sera from patients with primary rubella infections (showing titre increases by HI) were treated with DEAE-Sephadex 50 as described above. The dialysed eluates obtained by this method were tested for rubell -specific IgM and IgA-antibodies by the IF-technique described above. All 19 patients showed presence of IgA but only 14 of the patients showed presence of IgM-antibodies. HI as well as IF rubella antibodies were present only in the second serum sample of the serum pairs examined. The first serum sample was obtained 0–2 days following the rash.

Discussion

W have observed that the following details of the technique employed were of value with respect to removing unspecific binding of the test sera to the uninfected control cells: a) Cultivation of the rubellavirus-infected and the uninfected control cells for at least three days in medium without serum before harvesting the cells, and b)

preliminary incubation of the slides with rabbit serum, as described above.

Several investigators (4 7 8 10) have employed baby hamster kidney (BHK) cell lines for rubellavirus IF techniques. We have not been able to obtain specific and reproducible results using these cells.

It has previously been described that the sensitivity of the IF-techniques employed (for IgM antibody determination) could be increased by examination of serum fractions (obtained by sucrose density gradient centrifugation) instead of whole sera (1 4). We have found that the use of serum fractions obtained by DEAE-Sephadex 50 absorption of whole serum (13) produced satisfactory results.

We have demonstrated that the sensitivity of the IF-technique compared favourable with the sensitivity of the sucrose density gradient centrifugation. This was also observed by *F rghans et al.* (7)

Banatvala & Best (2) have advocated that, in cases of doubt, more than one technique should be employed for the detection of rubella IgM antibodies. It has been our experience that the two methods: sucrose density gradient centrifugation and IF-techniques, supplement each other to a certain degree with regard to detection of these antibodies.

We have confirmed the consistent appearance of IgA-antibodies in acute postnatal rubella infections, as demonstrated earlier by *Cradock Watson et al.* (4) We have furthermore found that the DEAE-Sephadex 50 serum fractions usually gav better and more consistent results (when IgA-antibodies were investigated) than the corresponding whole sera. The persistence of IgM and IgA-antibodies is being investigated presently by the method described

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HAEMOPHILUS APHROPHILUS SEPTICAEMIA

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Haemophilus aphrophilus was recovered from blood cultures from a 52-year-old woman with septicaemic disease including joint swelling and suspected endocarditis. The typical late and slight growth of the organism on bacteriological media is pointed out.

Haemophilus aphrophilus is a very rarely encountered, small Gram-negative bacterium closely related to *Actinobacillus actinomycetemcomitans*. Since *Khairat* (4) first described the organism in 1940 only 50 cases of *H. aphrophilus* endocarditis have been reported (3). The bacterium may be recovered on ordinary bacteriological media, but its growth is markedly slow and the macroscopic changes produced in fluid blood cultures may be very slight (2, the present case). These features may possibly partially account for the apparently extremely rare occurrence of *H. aphrophilus* infections. We therefore considered it of value to describe a recent case of septicaemia in which repeated, ordinary blood cultures yielded growth of organisms showing all the characteristic bacteriological properties of *H. aphrophilus*. Our patient presented joint swelling, a symptom which is not generally described as a typical feature of generalized *H. aphrophilus* infections.

CASE REPORT

A 52-year-old woman with no previous history of rheumatic fever or heart disease. She had a dental extraction in February 1972 and the following month she underwent a partial gingivectomia because of paradentosis.

On December 3rd 1972, acute swelling of the right ankle developed; four days later she had chills and fever 39° C, persisting for one week. Following institution of penicillin V the arthritis receded in 2-3 days and the fever gradually subsided. On December 29th she was admitted to Oslo City Hospital, surgical department Legevakten, with symptoms suggesting acute thrombophlebitis in the left leg and left ankle arthritis: the temperature was 39.2 C, ESR 85 mm, haemoglobin 9.8 g/100 ml, leucocyte count 9200/cu mm. During three days of phenylbutazone and penicillin V treatment, she became nearly afebrile. Another episode of chills and septic fever on January 5th 1973 was treated with 1 g of perroral tetracycline daily for four days without significant effect. Between January 9th and 15th, three blood cultures were made, all of which were sterile.

On January 16th she was transferred to the medical department Krohgstøtten after another episode of septic fever. On transfer she had arthritis in the first metacarpophalangeal joint on her right arm, and a maculopapulous exanthema around the wrist, but no other exanthema, petechiae or cutaneous bleedings. A soft systolic murmur grade III was heard in the 2nd intercostal space and the precordium, with a normal 2nd heart sound. Repeated blood cultures taken between January 17th and 21st now yielded growth of small Gram-negative rods. On January 22nd treatment was started with penicillin G 30 megaunits and cephalotin, 6 g daily intravenously. As there was no significant temperature response this treatment was replaced by streptomycin 2 g and co-

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phalotin 4 g daily from January 26th. A gradual improvement then took place. Ultimately cephalotin intravenously was replaced by a daily 4 g of peroral cephalexin. This therapy was discontinued on February 16th because of an allergic rash. The patient was discharged on February 20th, and recovered well.

In October 1973 the patient developed icterus and haematemesis. Laparotomy revealed a pancreatic carcinoma infiltrating the duodenum.

BACTERIOLOGICAL FINDINGS

Blood cultures were made by inoculating 5 ml venous blood into 50 ml nutrient broth. Out of 10 blood cultures made between January 17th and 21st, 6 yielded growth of identical organisms. Macroscopically the positive cultures showed a development of small, dry granules on the bottom layer of blood cells, the supernatant fluid remaining clear. The granules were initially difficult to distinguish from the sediment of white blood cells, but they increased slowly in size during prolonged incubation. Microscopic examination revealed their composition as aggregates of small Gram-negative rods.

Growth on solid media: on blood agar (containing defibrinated sheep blood) a very light growth of minute colonies was visible after one day's incubation, increasing slowly in size during incubation for up to one week. The colonies were adherent to the agar surface leaving indentations when removed, and they produced a faint alpha-haemolysis. Growth occurred both aerobically and anaerobically (i.e. in an atmosphere consisting of 95 per cent N_2 and 5 per cent CO_2); it was significantly better under the anaerobic condition. The aerobic growth on blood agar was clearly enhanced by carbon dioxide. Microscopically the cultures showed small Gram-negative rods, in some subcultures pleomorphism appeared, including long thread-like forms as well. A very sparse growth also took place on plain nutrient agar. No requirement for either X or V factor was observed.

In fluid media the strain grew predominantly as granules adhering to the walls of the tube: the growth was heavier in serum broth than in plain nutrient broth. The organisms were non-motile at 37° C and 20° C. No growth occured in solid or fluid media at 20° C, even after prolonged incubation.

Results of biochemical tests: Tests for urease, oxidase and catalase were all negative. Indol and hydrogen sulphide were not produced. Nitrate was reduced to nitrite. The strain fermented lactose, maltose, glucose and sucrose, producing acid. The organisms failed to ferment xylose and mannitol. (Fermentation reactions were rapid and clear-cut when tested on serum-agar, whereas tests in peptone water yielded inconsistent results.)

The sensitivity of the strain to antibiotics was tested by the *Ericsson* method (1). The isolate was found "Sensitive" to penicillin G, methicillin, ampicillin, sulphamodimidin, tetracycline, oxytetracycline, chloramphenicol, streptomycin, cephalotin, gentamicin, colistin, kanamycin and neomycin. The strain was "Fairly sensitive" to erythromycin and lincomycin and "Resistant" to fucidin and bacitracin.

DISCUSSION

The characteristics of the strain all conform with the authorative description of *Haemophilus aphrophilus* given by *King & Tatum* (5). A bacteriological diagnosis of *Haemophilus aphrophilus* bacteraemia depends firstly upon the recognition of its characteristically sparse and slow growth in blood culture bottles; secondly a possible presence of this extremely rarely encountered microorganism must be borne in mind during the diagnostic considerations. The diagnosis may then be established by routine methods. The differentiation of the organism from *Actinobacillus actinomycetemcomitans* rests upon the positive catalase test given by the latter together with distinctive differences in the sugar fermentation patterns, the examination of which may be critically influenced by the methods employed.

In a compilatory study (7) *Page & King* reported the clinical pictures in 41 patients from whom *H. aphrophilus* had been isolated,

17 of whom had bacteraemia and 24 had localized infections. Fifteen of the bacteraemic patients had endocarditis, in most cases associated with an underlying heart disease. In the present case, endocarditis was suspected from a systolic aortic murmur the diagnosis, however was not definitely settled. No underlying heart disease was known. Joint swelling, which was a predominant symptom in our patient, is not generally described as a characteristic feature in generalized *H aphrophilus* infections (7) the symptom was, however also present in the case reported by *Farrand et al.* (2)

Page & King (7) held that the clinical pictures of *H aphrophilus* infections strongly point to the mouth or the respiratory tract as the portal of entry *Kraut et al.* (6) cultivated the organism from human oral flora using a selective medium with bacitracin incorporated, and their results indicate that *H aphrophilus* is a component of the normal mouth flora in a substantial percentage of the adult population. In the present case, the patient underwent a rather extensive dental treatment for paradentosis prior to the *H aphrophilus* infection the interval, however was as long as 7–10 months. The paradentosis process itself may also be suspected to have played a role in the pathogenesis. Furthermore, the patient suffered from a pancreatic carcinoma which was unrecognized but obviously present at the time of her septicaemic disease.

The patient died from pneumonia on June 26, 1974 after having suffered from several episodes of septicaemia caused by various bacteria other than *H aphrophilus*. Post-mortem examination showed the pancreatic carcinoma infiltrating the duodenum with formation of ulceration. No changes in the heart valves consistent with endocarditis were observed.

We are grateful to Professor *Sverre Dick Henriksen*, Oslo, who discussed the diagnostic bacteriological problems and made valuable suggestions, and to Dr. *Robert E. Weaver* Special Bacteriology Unit, Center for Disease Control, Atlanta, Georgia, to whom the isolate was submitted for verification and who confirmed our diagnosis.

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THE SUSCEPTIBILITY OF AEROBIC AND ANAEROBIC BACTERIA, L-PHASE VARIANTS, CANDIDA, PROTOZOA AND VIRUSES TO LYSOLECITHIN

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The L-phase variants of three bacteria were at least 100-fold more susceptible to lysolecithin than their corresponding parents. Other bacteria, a yeast and protozoa were relatively resistant to lysolecithin although some bacteria, notably anaerobes, were particularly sensitive. It seems that some of the differences in sensitivity to lysolecithin may be accounted for, in part, by differences in the cholesterol content of the cell membranes and walls. Preliminary observations indicate that the infectivity of lipid-containing viruses, i.e. influenza B virus and *Herpesvirus hominis* type 1, but not of non-lipid-containing viruses, i.e. an adenovirus and a rhinovirus, was diminished by lysolecithin treatment. The possibility that lysolecithin production might interfere with the isolation of L-phase variants is discussed. Also considered is the possibility that the differential sensitivity of mycoplasmas and stable L-phase variants to lysolecithin might provide a means of distinguishing between them.

Tissue extracts often kill mycoplasmas so causing difficulty in their isolation. It seems that the formation of lysolecithin by the extracts is responsible for this, at least in part, since it damages the mycoplasma membrane (4, 5, 9, 10). Bacteria in the L-phase may also be difficult to isolate. Many of the features of these organisms are similar to those of mycoplasmas (13), one of these being an outer membrane containing phospholipids, which, presumably, is susceptible to the effect of lysolecithin. It was, therefore, of particular interest to determine the susceptibility of L-phase variants of bacteria to lysolecithin and to see how this compared with the susceptibility of their bacterial parents and of other bacteria. Finally, the outer coats of certain protozoa contain phospholipids as do the envelopes of some viruses and so we have tested the lysolecithin susceptibility of these different agents and discuss the implications of our findings in attempts to recover the organisms from tissues.

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MATERIALS AND METHODS

Micro-organisms

L-phase variants of bacteria. Strain G203U, a non-haemolytic L-phase variant of a *Streptococcus pyogenes* was obtained from Professor C. Lack. An L-phase variant 524 of *Staphylococcus aureus* was obtained from Professor R. E. O. Williams and L-phase L9 of *Proteus mirabilis* from Dr R. M. Lemcke.

Bacteria. The bacterial form of *S. aureus* (Oxford strain) was obtained from the N.C.T.C. (Colindale) and *Streptobacillus moniliformis* from the

A.T.C.C. (Washington, D.C.) Other bacterial forms were freshly isolated.

Fungus and protozoa. *Candida albicans* and *Trichomonas vaginalis* were freshly isolated. *Entamoeba histolytica* was received from Hoffman-La-Roche, Basle.

Viruses. Influenza-virus strain B/Taiwan/2/62 was obtained from Dr *A. S. Baars*. *Herpesvirus hominis* type 1 from Dr *A. C. Allison*; adenovirus type 3 from Professor *L. Kjellén* and rhinovirus type 2 (HGP) from Dr *E. J. Stott*.

Media

L-phase variants of *S. pyogenes* and *S. aureus* were grown in brain-heart-infusion broth (Difco) containing 10 per cent horse serum, 5.0 per cent NaCl and 100 U penicillin/ml, while that of *Proteus mirabilis* was grown in liquid mycoplasma medium (7). Solid media for colony-counting were prepared by incorporating 1.0 per cent agar in the media mentioned above. The bacterial forms corresponding to the L-phase variants were grown in Todd-Hewitt medium (13). Other bacteria were grown in tryptose broth (Difco) with the exceptions of *Clostridium perfringens* and an anaerobic streptococcus which were cultured in thioglycollate broth, and *Streptobacillus moniliformis* which was grown in nutrient broth (Difco) containing 20 per cent horse serum. *C. albicans* was grown in tryptose broth. Solid media of blood- and haematin agar were also used. *T. vaginalis* was cultured as described previously (8) while *E. histolytica* was propagated in tubes containing a slope of coagulated horse serum to which was added fresh horse serum diluted 1:6 in saline, one loop of rice starch and two of horse blood were added to each tube.

Tissue-culture Cells and Media

Primary monolayer cultures of calf kidney cells were prepared and maintained as described by *Baar & Kraut* (2). Primary cultures of human thyroid cells were prepared according to the method of *Pulvertaft et al.* (11). They were grown in Eagle medium containing 10 per cent calf serum, 3 per cent sodium bicarbonate and 100 U penicillin/ml; maintenance medium was the same but with the calf serum reduced to 1.0 per cent. MARA cells (6) originally derived from bone marrow were grown in Earle medium with 10 per cent foetal calf serum and maintained with 1.0 per cent serum. HeLa (Ohio) cells were grown and maintained as described by *Stott & Tyrrell* (14).

Tests for Susceptibility of Bacteria, Fungi and Protozoa to Lysolecithin

Lysolecithin was obtained from Sigma. Two-fold dilutions were made in the respective liquid media and an equal volume of the medium, containing not less than 10^4 organisms, was added. Mixtures of L-phase organisms and lysolecithin were incubated at 37 C and subcultures were made on L-phase medium after 4 and 24 hours of incubation. The plates were incubated for up to 3 weeks. Mixtures of lysolecithin and the bacterial species, corresponding to the L-phase variants, were inoculated on blood agar after 4 and 24 hours of incubation. The other bacterial species were seeded either on blood agar or haematin agar after 4 hours of incubation. *Streptobacillus moniliformis*, *Neisseria meningitidis* and *C. albicans* were incubated overnight at 37 C before subculture. In all these tests, cultures without lysolecithin were included. The cidal concentration of lysolecithin was the lowest dilution which caused complete inhibition of growth on subculture to solid medium. Cultures of *E. histolytica* and *T. vaginalis* were observed for motile trophozoites and trichomonads repeatedly over 48 hours, and the highest dilution of lysolecithin which caused complete inhibition of movement was recorded.

Test for Susceptibility of Viruses to Lysolecithin

Two-fold dilutions of lysolecithin were made in tissue culture maintenance medium and different numbers of virus particles, as indicated in Table 3 and below, were added and incubated at 37 C for 90 minutes. The mixtures were then inoculated into the appropriate tissue culture system. Influenza virus B/Taiwan/2/62 was assayed by plaque technique described by *Baar & Kraut* (2) and other viruses by their ability to cause cytopathic changes in tissue-culture cells. Concentrations of lysolecithin which inhibited virus plaque formation or cytopathic effect were recorded.

RESULTS

L-phase Variants and Corresponding Bacteria

The variants were much more susceptible to lysolecithin than the corresponding bacteria (Table 1). Thus, the L-phase variants were killed by 62.5 µg/ml or less of lysolecithin, while 250 µg/ml were required to kill *S. pyogenes* and the two other bacterial species were not killed by 1000 µg/ml.

Bacteria, a Yeast and Protozoa

The difference already seen (Table 1) in the susceptibility of different bacteria to lysolecithin was further noted when other bacteria were examined (Table 2). Some of the

TABLE 1 *The Susceptibility of L-phase Variants and the Corresponding Bacteria to Lysolecithin*

Organism tested	Growth at indicated concentration (μg/ml) of lysolecithin				
	1000	250	125	62.5	15 6
L phase variant (C203U)	—	—	—	—	—
Streptococcus pyogenes	—	—	+	+	+
L-phase variant (524)	—	—	—	—	+
Staphylococcus aureus	+	+	+	+	+
L-phase variant (L9)	—	—	—	—	+
Proteus mirabilis	+	+	+	+	+

TABLE 2. *The Susceptibility of Bacteria a Yeast and Protozoa to Lysolecithin*

Organism tested	Minimal cidal concentration (μg/ml) of lysolecithin
Cl. perfringens	1 0
Anaerobic streptococcus	<7.8
α-haemolytic streptococcus, *D pneumoniae N meningitidis*	15 6
S faecalis St eptobacillus moniliformis	250
E. coli (smooth strain) *E. coli* (rough strain) *Pr morgani, Ps. aeruginosa, Sarcina lut S epidermidis*	>500
C albicans	>500
E. histolytica T vaginalis	1000

TABLE 3 *The Susceptibility of Influenza Virus B to Lysolecithin*

Titre of virus suspensions (p.f u./ml) after 90 minutes incubation with indicated concentration of lysolecithin (μg/ml)		
0	100	200
6.6×10	1.5×10	—*
6.6×10^{3}	0.2×10^{1}	—
6.6×10	0	—

* Cell monolayers destroyed by this concentration of lysolecithin.

bacteria, namely an α haemolytic streptococcus, an anaerobic streptococcus, *D pneumoniae* and *N meningitidis* were all inhibited by 15 6 μg/ml of lysolecithin, and a strain of *Cl. perfringens* was particularly sensitive being inhibited by 1 0 μg/ml. On the other hand, several of the bacteria and *C albicans* were not killed by 500 μg/ml of lysolecithin.

In the presence of 500 μg/ml of lysolecithin motile trophozoites of *E histolytica* and *T vaginalis* were no longer observed, and at a concentration of 1000 μg/ml no protozoal organisms could be detected, indicating that they had lysed.

Viruses

Influenza virus B/Taiwan/2/62 appeared to be susceptible to lysolecithin. Thus, the number of virus infectious units contained in a suspension was reduced by more than 100-fold after contact with lysolecithin for 90 minutes at 37 C (Table 3) It is worth noting that 200 μg/ml of lysolecithin destroyed the calf kidney cells used for the virus titration. *Herpesvirus hominis* type 1 seemed also to be susceptible since 25 μg/ml of lysolecithin inhibited the cytopathic effect in thyroid cell monolayers produced by 10 TCD_{50}. Likewise 50 μg/ml of lysolecithin caused damage of these cell monolayers. Adenovirus 5 was not found to be susceptible to 20 μg/ml of lysolecithin in tests carried out in MASA cells however the latter were found to be susceptible to 30 μg/ml of lysolecithin. The cytopathic effect produced by 10 TCD_{50} of rhinovirus type 2 in HeLa cells was not affected by prior incubation of the virus with 50 μg/ml of lysolecithin 100 μg/ml of lysolecithin caused a non-specific cytopathic effect in the HeLa cells.

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GAS CHROMATOGRAPHY OF BACTERIAL WHOLE CELL METHANOLYSATES

II A Procedure for Fractionation and Identification of Fatty Acids and Monosaccharides of Cellular Structures

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A procedure for determination of the fatty acid and monosaccharide composition of bacterial whole cell preparations has been developed. Specific elution profiles of total fatty acids, hydroxy fatty acids and monosaccharides from 1–10 mg dry cells are obtained. The method is based on the following five steps 1 Selective depolymerization of washed, lyophilized whole cells by treatment with HCl in anhydrous methanol. 2. Trifluoroacetylation of released and solubilized monomeric compounds. 3 Fractionation of the trifluoroacetylated methanolysate by an acetonitrile/hexane two phase solvent system. 4 Analyses of the fractions by gas-liquid chromatography 5 Peak identification by retention characteristics, selective extraction, reduction, and by mass spectrometry

Analyses of chemical constituents of whole cells, cell wall preparations or extractable cellular components have been demonstrated to be valuable for bacterial characterization and classification (1 13 16, 27 33 35) Several classes of substances and a number of separation methods have been explored. Methanolysis of freeze-dried whole cells followed by gas-liquid chromatography (GLC*) of the released fatty acid methyl esters and methyl glycosides is a recent approach (18, 23 25 38) In our previous studies (8, 14 19) we employed GLC elution profiles of TFA derivatized whole cell methanolysates directly as bacterial "finger prints" These were useful as a taxonomic tool even without chemical identification of the eluted components. However for an exact qualitative and quantitative comparison of strains appropriate peak identification would be necessary This communication describes an extended procedure where methods for fractionation and identification of fatty acids and monosaccharides have been included.

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* The following abbreviations are used

AN = Acetonitrile
BSA = N,O-bis-trimethylsilyl-acetamide.
EGA = Ethylene glycol adipate.
GLC = Gas-liquid chromatography
MS = Mass spectrometry
TFA = Trifluoroacetyl.
TFAA = Trifluoroacetic anhydride
TMCS = Trimethylchlorosilane.
TMS = Trimethylsilyl

MATERIALS AND METHODS

Bacterial Strains

Neisseria gonorrhoeae 21319/70, *N meningitidis* M1 *N flavescens* ATCC 13120 *N ovis* 37/59 *Moraxella nonliquefaciens* NCTO 7784 N-a, and *M bovis* ATCC 10900 N-a were selected to illu-

strate methodological aspects. The strains have previously been studied by GLC (8, 14 19)

Media and Growth Condition

The methods of cultivation on blood agar medium and of cell harvesting were as described (19)

Methanolysis and TFA Derivatization

With a few modifications these two procedures were performed as before (19) Freeze-dried bacterial cells (1–10 mg) were methanolyzed at 85 C for 18 hours. When cool, the heterogeneous mixture was concentrated to about 1 ml by a stream of dry nitrogen at 40 C (to remov HCl) and then centrifuged for 15 minutes at 1000 × g After one washing of the residual material with 1 ml dry methanol, the pooled supernatants were concentrated to dryness by nitrogen or on a rotary evaporator t 25 C. Traces of HCl were removed by repeated additions of methanol (2 × 5 ml) and concentration to dryness. TFA derivatization was performed by addition of an equal volume mixture of TFAA and AN (250 µl) to the dry methanolysate. The reaction mixture was kept at 85 C (sandbath) for 5 minutes in a tightly closed vial and left at room temperature for 1–2 hours before "finger print GLC (profile 1) or further steps were undertaken, as outlined in Fig. 1

Separation f Fatty Acids and Monosac harides

The fatty acid methyl esters of the TFA derivatized methanolysates were extracted by hexane (3 × 1 ml) Phase separation was achieved by centrifugation in 10 ml glass tubes (1000 × g, 5 min) and the hexane phases collected with a syringe. The AN bottom phase was discarded or analysed further for monosaccharides (see below and Fig 1 left branch) After one washing with AN (50 µl) the pooled hexane extracts were concentrated to dryness and redissolved in AN (~50 µl) for fatty acid analyses (see below and Fig 1 right branch) The residue after washing with AN was discarded

Exchange f TFA with TMS Groups

Due to the complexity of the AN bottom phases, these mixtures were analysed both as TFA and TMS derivatives (Fig 1 left branch) Accordingly after the first profile of the AN bottom phase (profile 4 Fig. 6) was obtained, the TFA substituents were split off This was achieved by 0.5 N HCl in anhydrous methanol (85 C, 5 hours) HCl and methanol were then removed as described above. Subsequent TMS derivatization was performed by an equal volume mixture (250 µl) of BSA (with 1 per cent TMCS) and AN The reaction mixture was heated for 5 min at 85 C and left at room temperature for 1–2 hours before chromatography leading to profile 5 (Fig 7)

All sol ents employed were of *pro analysis* grade and distilled before use For n-hexane the term hexane is used

Gas-Liquid Chromatography

The GLC equipment has been described previously (19) The samples (1–5 µl) were injected "on column Nitrogen, with a flow rate of 30 ml/min, was used as carrier gas. A Hewlett Packard model 3370-B digital integrator calculated peak areas and recorded rentention times.

The TFA derivatized methanolysates were analysed on 10 per cent W982 (19) Fatty acid methyl esters were analysed on the following two columns 10 per cent W982 (unpolar) and 10 per cent ethylene glycol adipate (EGA) (Applied Science Lab. Inc.) (polar) The methyl glycosides were analysed as TFA derivatives on a 5 per cent SP 2401 (Supelco Inc.) or OV-210 (39) and as TMS derivatives on the W982 column. Gas-Chrom Q, 100–120 mesh (Applied Science Lab. Inc.) or Chromosorb W 100–120 mesh (AW DMCS HP) were used as support. The initial temperature was 120 C for fatty acid methyl esters and TMS derivatives, and 90° C when TFA methyl glycosides were present. Temperature was programmed at 2 C/min, but additional analyses

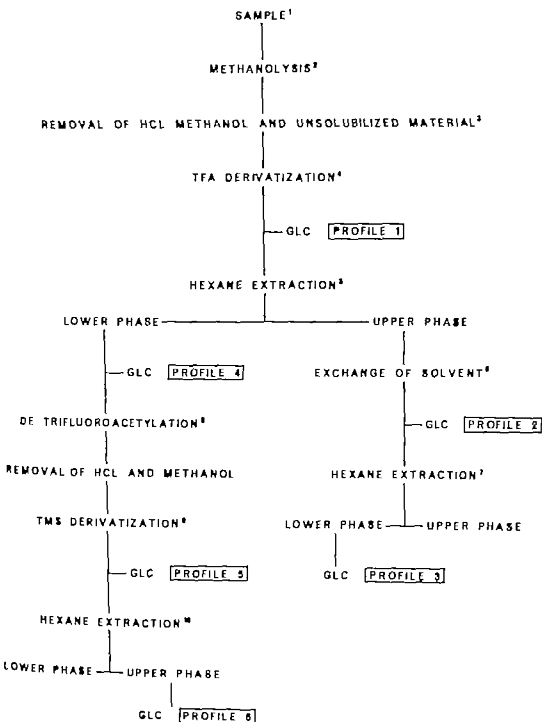
Fig 1 Flow scheme f the procedure The gas chromatographic conditions are outlined in the text.

Profiles 1 "finger print" 2, "total fatty acids 3 "polar fatty acids 4 "TFA monosaccharides ; 5 "TMS monosaccharides 6 "TMS neutral monosaccharides"

Freeze-dried bacteria, 1–10 mg.

- 2N HCl in methanol, 3 ml 18 hours, 85 C, N -atm.
  N_2, 40° C, centrifugation.
  TFAA/AN (1 1) 250 µl, 5 min, 85 C.
  AN-saturated hexane, 3 × 1 ml, AN-wash, 50 µl centrifugation.
- From hexane to AN 250 µl.
- AN-saturated hexane, 3 × 50 µl, centrifugation.
- 0.5N HCl in methanol, 1 ml, 3 hours, 85 C. (BSA + 1 per cent TMCS)/AN (1:1) 250 µl, 5 min, 85 C.
- AN-saturated hexane, 3 × 200 µl AN-wash, 50 µl, centrifugation.

Abbreviations AN = Acetonitrile BSA = N O-bis-trimethylsilyl-acetamide GLC = Gas-liquid chromatography TFAA = Trifluoroacetic anhydride TFA = Trifluoroacetyl, TMS = Trimethylsilyl, TMCS = Trimethylchlorosilane.


SAMPLE[1]
METHANOLYSIS[2]
REMOVAL OF HCL METHANOL AND UNSOLUBILIZED MATERIAL[3]
TFA DERIVATIZATION[4]
GLC
PROFILE 1
HEXANE EXTRACTION[5]
LOWER PHASE
UPPER PHASE
GLC
PROFILE 4
EXCHANGE OF SOLVENT[6]
DE TRIFLUOROACETYLATION[8]
GLC
PROFILE 2
REMOVAL OF HCL AND METHANOL
HEXANE EXTRACTION[7]
TMS DERIVATIZATION[9]
LOWER PHASE
UPPER PHASE
GLC
PROFILE 5
GLC
PROFILE 3
HEXANE EXTRACTION[10]
LOWER PHASE
UPPER PHASE
GLC
PROFILE 6

were performed isothermally at several temperatures.

Peak Identification

Fatty acids were primarily identified by comparison of their retention times with those of standards (Applied Science Lab. Inc., Analabs Inc., and Supelco Inc.) Hydroxy fatty acid methyl ester standards were TFA derivatized as described above. Both unpolar and polar columns were employed (see above) The polarity of each component was elucidated as shown in the right branch of Fig 1 Dissolved in AN the mixture was washed with small volumes of hexane (3 × 50 µl) Comparisons of elution profiles (profiles 2 and 3 Fig 5) before and after this selective extraction step provided the relative polarity of each component. These partition characteristics can be quantitatively expressed by p-values* determined as described below As supplements to the flow chart procedure (Fig 1) other methods were employed for further peak identification. Thus, unsaturation was detected by reduction (7) Cyclopropane groups were revealed by careful hydrogenation and subsequent bromination (6) Presence of hydroxyl groups was confirmed by rechromatography after removal of the TFA groups.

Monosaccharides (as TFA methyl glycosides) remained in the AN phase after hexane extraction. GLC analysis of this fraction led to profile 4 (Fig 6) Subsequent characterization was carried out according to the flow chart of Fig. 1 After removal of TFA groups, followed by TMS derivatization (see above) GLC analysis yielded profil 5. An additional profile where neutral monosaccharides predominate (profile 6) was obtained as follows the TMS derivatized mixture was extracted by hexane (3 × 200 µl) then the hexane extract was washed with AN (50 µl) concentrated to dryness, redissolved in hexane (200 µl) and chromatographed as before Retention times and peak configurations were compared to those of standard monosaccharides (Sigma Chemical Co.) which had been methanolyzed and derivatized by the given procedure

Mass Spectrometry

Structural assignments were confirmed by mass spectrometry A Varian MAT CH 7 gas chromatograph/mass spectrometer combination instrument was used, working at 70 eV with an electron current of 300 µA an accelerating voltage of 3 kV a molecule separator temperature of 250 C, and an ion source temperature of 190 C The fragmentation patterns obtained were compared to literature values (9 12, 24 30 31) and to those obtained from standards.

*The fractional amount partitioning into the nonpolar phase of an equal volume two phase solvent system" (5)

p-Value Determination

A micromethod modification of the procedure of *Bowman & Beroza* (5) was employed. The AN/hexane binary solvent system was used, both solvents saturated with the other prior to extraction. The solute, or mixture of solutes, was dissolved in 250 µl AN and a 2 µl aliquot was analysed by GLC. Then 250 µl hexane was added and the phases thoroughly mixed by vigorous vibration. After centrifugation the AN phase was re-analysed (see also *Selective Extraction in Peak Identification* below)

RESULTS AND DISCUSSION

The procedure described comprises liberation of fatty acids and monosaccharides from polymers and their subsequent fractionation and GLC analysis. Strictly anhydrous conditions are kept during the procedure to avoid degradation of fragile structures. The method represents a modification and extension of our previous GLC technique (19) and it has been worked out on freeze-dried bacterial cells. To a limited extent it has also been successfully applied to extractable lipids and purified lipopolysaccharides (unpublished results) In principle the procedure could be utilized for analysis of fatty acids and carbohydrates of any complex biological material. Various aspects of the procedure will be discussed in the following

Depolymerization

Fatty acids of bacterial lipids are most commonly determined by treatment with dilute alkali in aqueous alcohol (saponification) followed by acidification, solvent extraction, esterification and GLC. Such a procedure is selective, but has for our purpose two important limitations. Firstly monosaccharides and to some extent 3-hydroxy fatty acids (28 29) are degraded by alkali treatment. Secondly extraction from aqueous alcohol becomes tedious and difficult to carry out quantitatively in the presence of hydroxy fatty acids. These compounds have

limited solubility in the unpolar solvent, and emulsification takes place.

In contrast, depolymerization of lipids by a trans-esterification reagent (e.g. anhydrous methanolic HCl) liberates fatty acids as methyl esters. Furthermore, this reagent does *not* degrade the released monosaccharides which are stabilized as methyl glycosides. Even fragile structures like N acetylneuraminic acid are stable in 2 N methanolic HCl at 85 C (10)

TABLE 1 *p-Values* of Some Common Bacterial Fatty Acid Methyl Esters in Acetonitrile/Hexane*

Fatty acid methyl ester‡	p-Value*
10:0	.67 (.58)†
12:0	77 (73)
14:0	.84 (.81)
14:1[1]	75
15:0	.87
16:0	.89 (.89)
16:1	.82 (.81)
17:0	.92
17:0-c[3]	.88
18:0	.94 (.95)
18:1	.87 (.88)
18:2[5]	.85 (.81)
19:0	.96
19:0-c[6]	.93

*The fractional amount partitioning into the nonpolar phase of an equal volume two phase solvent system (5) See text for experimental details and discussion

‡ The figure before colon indicates number of carbon atoms in the chain, the one after colon denotes the number of double bonds in the methyl ester; -c indicates cyclopropane groups.

[1]methyl cis-9-tetradecenoate, [2]methyl cis-9-hexadecenoate, [3]methyl cis-9 10-methylenehexadecanoate, [4]methyl cis-9-octadecenoate, [5]methyl cis,cis-9,12-octadecadienoate, [6]methyl cis-9 10-methyleneoctadecanoate

† Values in parentheses are from *Bowman & Beroza* (5)

No degradation of the common bacterial fatty acids specified in Tables 1 and 2 could be detected after treatment with anhydrous methanolic HCl, except in the case of cyclopropane fatty acids. These were in our hands largely disintegrated by methanolysis, only some ten per cent remaining undegraded.

TABLE 2. *p-Values* of Some Bacterial Hydroxy Fatty Acid Methyl Esters and TFA Derivatized Methyl Glycosides in Acetonitrile/Hexane*

Compound	p-Value*	
	TFA derivatized‖	Underivatized
Methyl ester		
3-OH 10:0†	.23	.09
2-OH 12:0	48	.33
3-OH 12:0	.37	.24
2-OH 14:0	.61	46
3-OH 14:0	.50	.37
2-OH 16:0	74	60
3-OH 16:0	.63	49
Methyl glycoside§		
D-ribose	<0.1	
D-glucose	<0.1	
D-glucosamine	<0.1	

* See corresponding footnote of Table 1

‖ See text for experimental details. TFA = Trifluoroacetyl.

† The first figure denotes the position of the hydroxyl group. The figure before colon indicates number of carbon atoms in the chain and the one after colon the number of double bonds. All fatty acid methyl esters used were commercial standards except 3-OH 16:0 which was isolated from *Nasseri elongata* M2 (see ref 20)

§ Standard monosaccharides methanolyzed and TFA derivatized according to the procedure given in the text.

Consequently alkaline hydrolysis would be necessary for the determination of such fatty acids. It should also be noted that considerable loss of methyl esters with carbon chains up to C_{12} occurs during concentration prior to GLC due to their high volatility (see also ref 57)

Extraction of Fatty Acid Methyl Esters

GLC of the TFA derivatization mixture (profile 1 Fig 3) yields useful "finger prints" of bacteria (8, 14 19) but because of the complexity the differences between elution profiles are difficult to evaluate. The main chemical constituents contributing to this profile seem to be TFA methyl glycosides and fatty acid methyl esters. Obviously interpretation and quantitation would be much easier with separate GLC profiles of

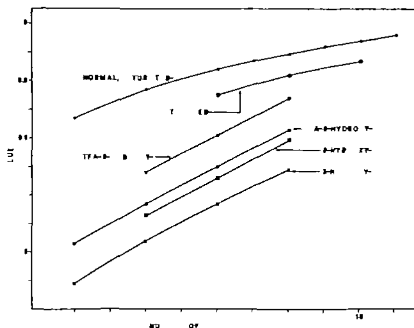


Fig 2 p-Values of fatty acid methyl esters versus chain length p-Value "The fractional amount partitioning into the nonpolar phase of an equal volume two phase solvent system" (5) Solvent system: acetonitrile/hexane. The experimental conditions are outlined in the text. All methyl esters used were commercial standards except 3-OH 16:0 which was isolated from *Veiuerla elongata* M2 (see ref. 20) TFA = Trifluoroacetyl.

the two classes of substances. Such a separation could readily be achieved by hexane extraction of the fatty acid methyl esters, as TFA methyl glycosides were found to be practically insoluble in hexane (Table 2) This extraction (see *Materials and Methods*) yielded a fatty acid methyl ester top fraction devoid of TFA methyl glycosides (profile 2, Figs. 3 4 and 5) Similarly the corresponding AN bottom phase did not contain fatty acid methyl esters in detectable amounts. The completeness of this extraction corresponds to the partition characteristics as seen from the p-values (see below) of Tables 1 and 2.

Thus, fatty acid methyl esters and TFA methyl glycosides can be separated completely and rapidly (no emulsification) by this anhydrous binary solvent system.

GLC of Methyl Glycosides

Derivatization of carbohydrates prior to GLC is mostly accomplished by TMS donor reagents (11 34) alditol acetates are used in structural studies (4 32) but other derivatives have not gained particular interest. However trifluoroacetylation of carbohydrates as described by *Vilkas et al.* (36) yields derivatives with very different properties they have a high polarity (see below) and are remarkably volatile. Thus, TFA hexosides elute from a methyl silicone column at about 90 C, which is 60–70 degrees below the eluting temperature of the corresponding TMS derivatives. Accordingly mixtures of TFA derivatized monosaccharides and long chain fatty acid methyl esters may be separated and analyzed on the same column (39 see also Fig 3)

The GLC analysis of the AN phase after extraction of the fatty acid methyl esters (Fig 1 left branch) involves a trifluoropropyl silicone (SP 2401 or OV-210) as stationary phase. This is reported to be highly selective for TFA derivatized monosaccharides (39) Although the column separates all isomeric ring forms of common pentoses, hexoses and hexosamines as TFA methyl gly

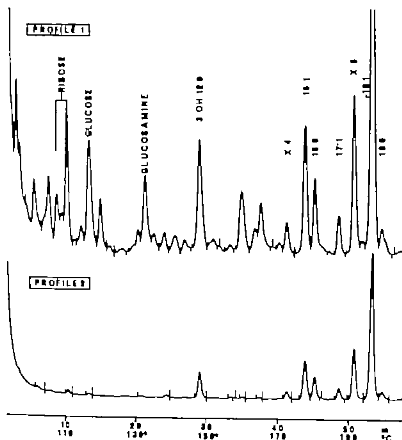


Fig. 3 "Finger print" profile (upper) and "total fatty acid" profile of Moraxella nonliquefaciens *7784* V-a (see Fig. 1 and the text for explanation) For the purpose of comparison, the starting temp. for profile 2 of this figure was 90° C and not 120° C as specified in the text. Other conditions were as described for the unpolar column (10 per cent W982) For explanation of symbols, see footnotes to Tables 1 and 2 and ref. 20.

cosides (39) profile 4 (Fig 6) is very complex with many unresolved peaks. In addition to TFA methyl glycosides, the AN phase might contain other polar bacterial constituents such as amino acid methyl esters, amines and alcohols. Consequently only the major monosaccharides may be determined by this semiquantitative procedure. For less complex mixtures, on the other hand, such as methyl glycosides of glycolipids (3 39) or lipopolysaccharides, the resolution is satisfactory and profile 4 is of high value for both identification and quantitation of monosaccharides (unpublished results)

Stronger evidence for peak identity is obtained when the samples are derivatized by different agents and analysed on different GLC columns. Accordingly exchange of TFA ester groups with TMS ether groups has been incorporated in the procedure (Fig 1 left branch) This is easily achieved in the same tube and without loss of material (see *Materials and Methods*)

Several silylating reagents for monosaccharides are available (26) most of these containing pyridine which has an unpleasant odour and unfavourable properties for GLC such as low volatility and a marked tailing A deleterious influence of this solvent on polyester liquid phases has also been reported (2) We use BSA in AN with TMCS as catalyst for silylation of monosaccharides.

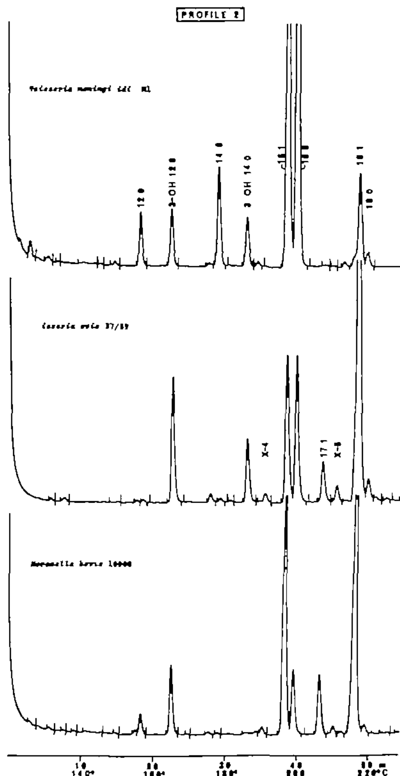


Fig 4 "Total fatty acid" profiles of th ee species of Neisseria *and* Moraxella. See Fig. 1 footnotes to Tables 1 and 2, text and ref 20 for explanation.

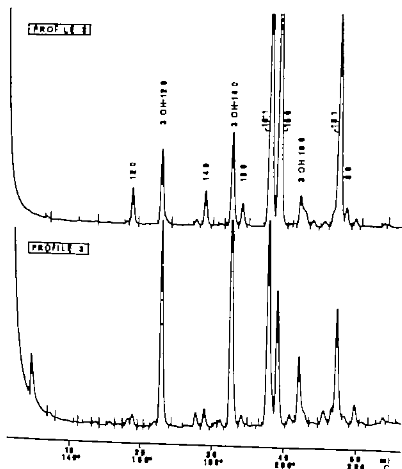


Fig. 5. Elution profiles of fatty acid methyl ester before and after selective extraction. "Total fatty acid" profile (upper) and "polar fatty acid" profile of *Neisseria flavescens* ATCC 13120. See text, Fig. 1 and footnotes of Tables 1 and 2 for explanation.

Hurst (17) recently showed this reagent to yield the most complete silylation of hexosamines in a comparative study of TMS donors. Acetonitrile is more volatile than pyridine, and the AN/hexane binary solvent system may be used for the fractionation of TMS derivatized mixtures (see below).

Selective Extraction in Peak Identification

Bowman & Beroza (5) showed GLC detection of partition characteristics to be a valuable tool for the identification of fatty acid esters and other organic compounds. They could distinguish closely related fatty acid esters by p-values (for definition see Table 1).

Re-extraction of the methyl esters (dissolved in AN) with small volumes of hexane (Fig. 1, right branch) removes the fatty acid esters in quantities corresponding to their p-values (Tables 1 and 2). This leads to an AN-phase which is highly enriched in the more polar TFA hydroxy acid esters (Fig. 5). Fig. 5 also demonstrates the marked rise in the concentration ratios of unsaturated to saturated methyl esters after such a selective extraction.

Fig. 2 illustrates the distinction of closely related hydroxy fatty acid esters by p-value determination. The effect of unsaturation and chain length on the partitioning in this solvent system is likewise apparent (see also ref. 5).

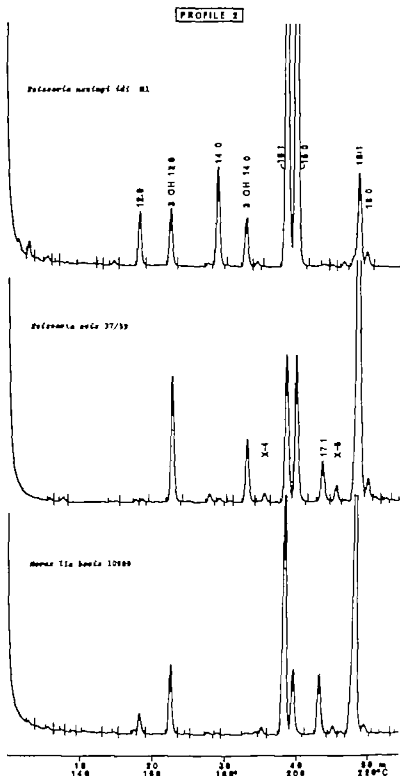


Fig 4 "Total fatty acid" profiles of th ee species of Neisseria *and* Moraxella. See Fig 1 footnotes to Tables 1 and 2, text and ref. 20 for explanation.

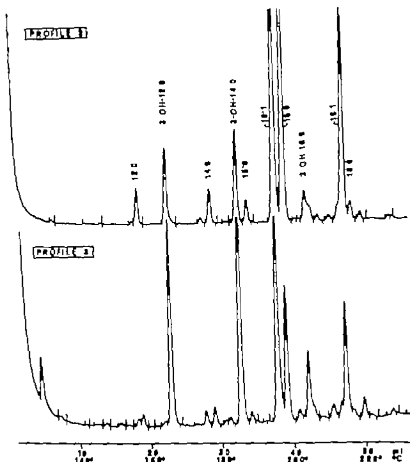


Fig. 5 Elution profiles of fatty acid methyl ester before and after selective extraction. "Total fatty acid" profile (upper) and "polar fatty acid" profile of *Neisseria flavescens* ATCC 13120. See text, Fig. 1 and footnotes of Tables 1 and 2 for explanation.

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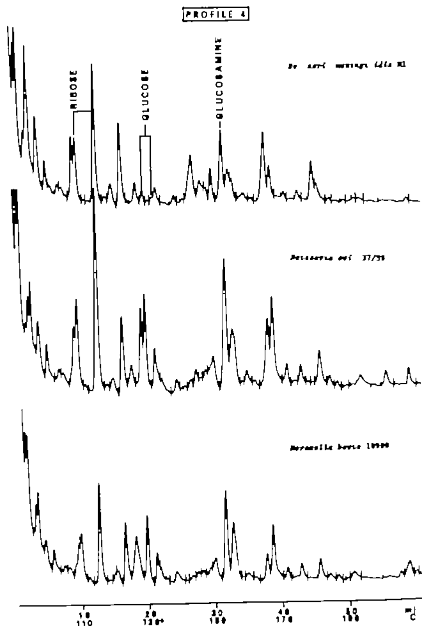


Fig 6 "*TFA monosaccharide profile of three species of* Neisseria *and* Moraxella. See text and Fig. 1 for explanation.

The significance of utilizing such procedures for characterizing compounds in the fatty acid fraction can be illustrated by two examples. 1) In the analysis of one strain of *Acinetobacter* we found that one of the fatty acids showed an unusually high preference for the AN phase, being more polar than TFA hydroxy fatty acid methyl esters eluting in the same region. The fact that this was a TFA dihydroxy fatty acid ester was confirmed by mass spectrometry (to be published). 2) In *Moraxella nonliquefaciens* two of the components of the fatty acid fraction were considerably less polar than the methyl esters having similar retention times. This observation was confirmed by mass spectrometry. No ester groups were detected from the fragmentation spectra. The taxonomic

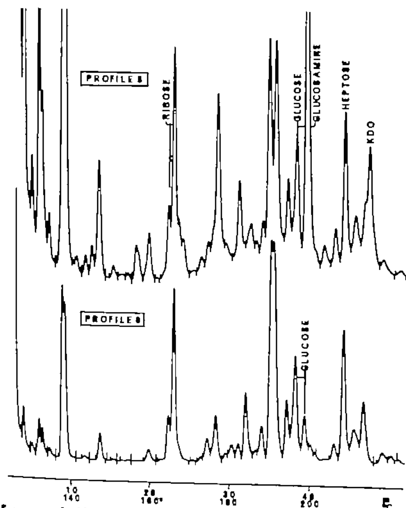


Fig 7 *"TMS monosaccharide" profiles befor and after selective extraction.* "TMS monosaccharide profile (upper) and "TMS neutral monosaccharide profile of *Neisseria gonorrhoeae* 21319/70. See text and Fig. 1 for explanation and discussion. KDO = 2-keto-3-deoxyoctonate.

significance of these components is demonstrated in a subsequent report (20) and their final identification is in progress.

Selective extraction has also been utilized for characterization of methyl glycosides (Fig. 1 left branch) Neutral methyl glycosides are almost quantitatively extracted into hexane, because of the nonpolar character of the bulky TMS groups. On the other hand, compounds with polar group(s) not being TMS derivatized are more likely to remain in the AN phase. This is the case of acidic and basic substances such as phosphate esters and amino acid methyl esters. The marked difference between profiles 5 and 6 (Fig 7) illustrates the utility of this extraction step The complete disappearance of the large glucosamine peak from the chromatogram (Fig. 7) was not expected, since the conditions of silylation used have been reported to yield a complete derivatization of hexosamines (17) Unintentional hydrolysis of the labile N TMS bond during the concentration step and unmasking of the polar amino group, may be the reason. Although not fully understood, this reproducible elimination of the glucosamine peak adds substantially to the identification of methyl glycosides.

Reproducibility and Taxonomic Implications

Factors like composition of growth medium, degree of aeration, temperature and the age of cells all have an influence on the composition of bacterial lipids (1 13 22) Nevertheless, with careful standardization of growth conditions, harvesting and analytical procedures, the determinations of bacterial fatty acids hitherto reported seem reasonably reproducible. Our previous results (19*) indicated a small variation in peak heights (standard deviation below 5 per cent) when the same batch of medium was used, and significantly more variation (up to 16 per cent) with different batches of medium. However variations in the quantity of individual fatty acids were of little consequence for the numerical affinity of a strain to groups of similar organisms (20) *Uchida & Mogi* (35) reported negligible variation (standard deviation below 2 per cent) for ten major fatty acids of *Pediococcus* at standard conditions.

Representative fatty acid profiles of neisseriae and moraxellae are shown in Figs. 3 (lower part) 4 and 5 The chromatograms have well resolved peaks and group-specific traits (20) The monosaccharide profiles (Figs. 6 and 7) are less clear-cut, but may provide a valuable taxonomic supplement, especially when more distantly related organisms are compared.

Cellular fatty acid composition determined by the procedure outlined discriminates well between different organisms grouped on the basis of genetic characteristics such as DNA base composition, and affinity in nucleic acid hybridization and genetic transformation (20, 21) Furthermore, strains of the same species most often have a rather similar fatty acid composition (20 21) These findings add to the impression of methodological reliability and demonstrate the feasibility of the procedure in taxonomic studies.

* This paper included an estimation of time to time variation in the TFA "finger prints" Seven abundant peaks in the elution profile of *Neisseria catarrhalis* Ne 11 were studied. Peak no. 5 has now been identified as methyl hexadecenoate (16 0) peak no. 6 as methyl n-hexadecanoate (16 1) and peak no. 7 as a mixture of methyl octadecenoate (18 1) and methyl octadecadienoate (18 2)

The mass spectral analyses were performed at the Institute of Clinical Biochemistry University of Oslo, Rikshospitalet, Oslo. The valuable assistance of Dr *E. Jellum* and Mr *P Helland* of that Institute is gratefully acknowledged.

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GAS CHROMATOGRAPHY OF BACTERIAL WHOLE CELL METHANOLYSATES

I. *Fatty Acid Composition of Neisseriae and Moraxellae*

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Strains of "true neisseriae" (*Neisseria gonorrhoeae*, *N. meningitidis*, *N. cinerea*, *N. flavescens* and the rodshaped *N. elongata*), "false neisseriae" (*N. catarrhalis*, *N. ovis* and *N. caviae*) and moraxellae (*Moraxella nonliquefaciens*, *M. bovis*, *M. lacunata*, *M. osloensis*, *M. phenylpyruvica*, *M. kingae* and the tentatively named *M. urethralis*) were analysed for contents of fatty acids. Thirteen fatty acids were identified. Differences in relative amounts of these and additional unidentified components allowed separation, by numerical analysis, of the strains in groups corresponding to genetic affinities. Thus, "true" and "false neisseriae" formed two distinct groups. The fatty acid composition of the rodshaped *N. elongata* was almost indistinguishable from that of *N. flavescens*, whereas moraxellae in this respect generally resembled "false neisseriae". Exceptions were *M. kingae* and *M. urethralis*, which both differed considerably from the other organisms studied. The utility of fatty acid analysis in identification of clinical isolates of these bacteria is indicated.

Characterization of neisseriae and moraxellae by gas-liquid chromatography (GLC) has been reported by us previously (9, 11, 16). It was demonstrated that GLC profiles of trifluoroacetyl (TFA) derivatized whole cell methanolysates rendered "finger prints" which reflected group relationships established by nucleic acid homology. Due to the complexity of the profiles, peaks with similar retention in different "finger prints" were not easily distinguished and identified. Accordingly the GLC procedure was supplemented by selective extractions and various other peak identification techniques (17). The present paper reports the application of the improved procedure in identification and quantitation of fatty acids in neisseriae and moraxellae. Numerical analysis is introduced as an aid in taxonomic evaluation of the fatty acid patterns.

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MATERIALS AND METHODS

Bacterial Strains

The strains examined are listed in Table 1. Except for *Moraxella lacunata*, all strains have been included in our previous GLC studies (9, 11, 16). In those reports, the genetic affinities of most strains have been discussed. They are grouped accordingly in Table 1. *Neisseria flavescens* ATCC 13120 and NCTC 8263 are treated as different strains, although they are not so, *sensu stricto*, since they are descendants of the same original culture. This is also the case of *N. caviae* ATCC 14659 and NCTC 10293 (23). The type strain of *M. lacunata*, ATCC 17967, has been described by Henriksen (12) and was received from him. The reference strain for the biotype *liquefaciens* of *M. lacunata* NCTC 7911 has been characterized and its genetic relations determined by Bøvre (3). *M.*

kingae is the corrected designation of *M. kingii* (7)

Growth Conditions and Harvesting

All strains examined were cultivated on blood agar for 20 hours in a humid atmosphere and harvested as described previously (16) *N. gonorrhoeae*, *N. meningitidis*, *N. flavescens* and *N. elongata* were grown at 37 C with initial CO_2 supplement and the remaining strains at 33 C without CO_2. See also the section of Reproducibility under Results.

Chemical Procedures

Extraction, derivatization and gas-liquid chromatography have been described in the preceding paper of this series (17)

Fatty Acid Analysis

The procedure for the fatty acid analysis has been detailed in part IV of this series (17). Bacteria were lyophilized and methanolyzed by 2 N HCl in anhydrous methanol. After removal of the reactants, free hydroxy groups of fatty acid methyl esters (as well as methyl glycosides) were derivatized by trifluoroacetic anhydride (TFAA). Subsequently the methyl esters were selectively extracted with hexane. Aliquots were analyzed by GLC on a polar and an nonpolar column. Peak areas were calculated by a digital integrator (17) and the percentage of the total fatty acids was determined for each peak (Table 1). For peak identification, a combination of several methods were used (for details see ref. 17). The linear relationship between the logarithm of retention time and chain length among homologous fatty acid methyl esters was used for primary characterization. Further identification was achieved by selective extraction and by reduction. Chemical structures were confirmed by mass spectrometric analysis.

Procedures of Numerical Analysis

The data matrix of fatty acid concentrations (Table 1) was evaluated by numerical analysis. The analysis employed the transformed Yule correlation coefficient (1) combined with unweighted pair group cluster analysis (25). Before calculation of the similarity matrix of primary elements (Table 3) the data matrix was transformed by the formula $y = ln\ (\ + 1)$ where x is an element in the data matrix as listed in Table 1 and y is the corresponding element in the new matrix. Fatty acid concentrations indicated in Table 1 by trace quantity (tr) for this purpose were given the value 0.2 per cent. By the logarithmic transformation, small quantities attain relatively more weight in correlation coefficients. Unweighted pair group cluster analysis based on the similarity matrix of primary elements, rendered the phenogram in Fig. 3. The matrix was also transformed to a group matrix of affinity (Table 4) by calculating the mean transformed Yule correlation coefficient $\bar{S}_{T}$, mean deviation from mean, $\bar{\Delta}$, and standard deviation, σ of the primary similarity coefficients. For this calculation were used indices with one digit.

RESULTS

Fatty Acid Composition

The fatty acid content of the 28 strains is discernible from Table 1. Eleven of the 19 compounds tabulated were identified by comparing retention times and mass spectrometric fragmentation patterns with those of standards. For 3-OH 16:0 and 17:1 (see footnote to Table 1 for fatty acid nomenclature) no standards were available. Conse-

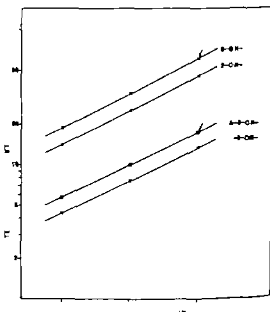


Fig. 1. Retention times of 2- and 3-hydroxy fatty acid methyl esters (underivatized and trifluoroacetyl (TFA) derivatized) versus number of carbon atoms in chain. The retention times are plotted in logarithmic scale. Column: 0.2 × 200 cm (glass) of 10 per cent ethylene glycol adipate on Gas-Chrom Q, 100–120 mesh. Temp.: 200° C. Flow rate 30 ml/min. Gas chromatograph: Hewlett Packard 5750 equipped with flame ionization detector.

The retention times of 3-OH 16:0 from *N. elongata* M12 are marked by arrows.

quently their identification was somewhat less conclusive.

The hydroxy fatty acid, 3-OH 16:0 was identified in the following manner

1) Chain length and presence of a hydroxy group were indicated by GLC retention times and comparisons of the GLC profiles before and after selective extraction (ref 17 Fig. 5)

2) The GLC properties of the unknown component and the reference hydroxy fatty acids were compared (Fig 1) The unknown component fits on the line of 3-OH fatty acid methyl esters, indicating a straight chain C_{16} fatty acid with a hydroxy group in 3-position.

3) Partition values (p-values) in the acetonitrile/hexane two phase system similarly indicated the hydroxy group in 3-position, and a straight, saturated C_{16} chain (ref 17 Table 2 and Fig. 2)

4) Mass spectrometric analysis confirmed the evidence obtained by GLC retention times and p-value determination. The mass spectra were compared to those of the references. In all of the 2-OH esters, the molecular ion and a peak at m/e 90 could be detected (24) The mass spectrum of the unknown (Fig. 2) lacks these two fragments. The base peak of the spectrum, m/e 103 is characteristic of 3-OH acid methyl esters and the peak at m/e M 50 has originated by loss of methanol and water from the molecular ion (24) Otherwise the fragmentation patterns were similar to those obtained from 3-OH 12:0 and 3-OH 14:0 methyl ester standards both as TFA derivatives and underivatized.

The identity of the 17 1 fatty acid was supported by the following evidence

1) On both columns, the retention time differed from that of 17:0 as expected from a monounsaturated C_{17} straight chain fatty acid methyl ester

2) Reduction with hydrogen gas in the presence of Pt on charcoal (2) and rechromatography resulted in a new peak with properties identical to methyl heptadecanoate (17:0)

3) The mass spectrum exhibited a molecular ion at m/e 282 and a fragmentation pattern resembling that of methyl palmitoleate (16 1)

Six components have not yet been identified (x's in Table 1) Mass spectrometric analysis showed x-4 and x 5 to be structurally

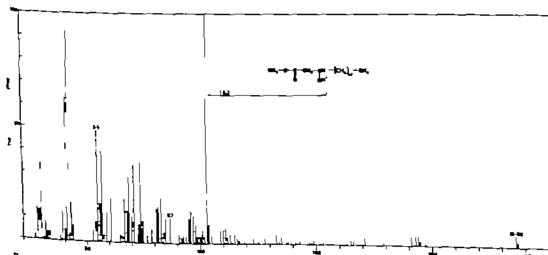


Fig 2 *Mass spectrum of methyl 3-hydroxyhexadecanoate (3-OH 16:0) from* Neisseria elongata M2 Gas chromatography/mass spectrometry analyses were done using a 0.2 × 200 cm glass column containing 10 per cent W982 or 10 per cent EGA on 100/120 mesh Gas-Chrom Q A Varian MAT CH 7 gas chromatograph/mass spectrometer combination instrument operating with an ionizing voltage of 70 eV was used. For further details see ref. 17

TABLE 1 *Fatty Acid Conten*

Group/Strain§		Fatty acid†‡						
		x 1	12:0	3-OH 12:0	x 2	x 3	14:0	3-OH 14:0
Group I								
N gonorrhoeae	21319/70	–	4	10	–	–	3	8
N gonorrhoeae	562/71	–	3	5	–	–	3	6
N meningitidis	M1	–	3	4	–	tr	7	3
N meningitidis	B8152/66	–	4	8	–	tr	7	6
N cinerea	159/62	–	3	5	–	–	2	4
N flavescens	ATCC 13120	tr	3	8	–	tr	3	9
N flavescens	NCTC 8263	tr	4	8	–	tr	3	8
N elongata	M2	–	2	6	–	tr	5	5
Group II								
N catarrhalis	Ne 11	tr	2	9	–	tr	tr	tr
N catarrhalis	13074/62	tr	1	6	–	tr	tr	tr
N ovis	199/55	tr	tr	9	–	tr	tr	4
N ovis	37/59	tr	tr	10	–	1	tr	3
N caviae	ATCC 14659	tr	1	4	–	tr	tr	3
N caviae	NCTC 10293	–	2	5	–	tr	tr	5
Group III								
M nonliquefaciens	4663/62	–	1	8	1	–	tr	–
M nonliquefaciens	NCTC 7784 N	–	tr	4	–	tr	tr	–
M nonliquefaciens	3067/66 N-a	–	tr	8	tr	1	1	–
M bovis	ATCC 10900 N-a	tr	2	7	–	tr	tr	–
M bovis	4 N-b	–	–	7	–	tr	–	–
M lacunata	ATCC 17967	–	1	4	–	–	tr	2
M lacunata (biotype *liquefaciens*)	NCTC 7911	–	1	7	–	–	–	4
Group IV								
M osloensis	A1920	–	–	8	–	–	–	3
M osloensis	5873	–	–	5	–	tr	–	3
Group V								
M phenylpyruvica	2863	2	tr	10	–	1	tr	–
M phenylpyruvica	ATCC 17958	–	1	35	–	–	tr	–
Group VI								
M kingae	4177/66 N-a	–	4	7	–	2	46	4
M kingae	9076/70 N-a	–	5	5	–	3	40	4
Group VII								
M urethralis	WM6	–	–	–	–	tr	4	7

* See text and the preceding paper (17) for experimental detail

§ N = *Neisseria* M = *Moraxella* *M kingae* is corrected from *M kingii* (7) *M urethralis* is a tentative designation (19) *N flavescens* ATCC 13120 and NCTC 8263 are descendants of the same original culture as are also *N caviae* ATCC 14659 and NCTC 10293 (23) Group I true neisseriae ; group II "false neisseriae" group III classical moraxellae

† Key to fatty acid designations the figure before colon indicates number of carbon atoms in the fatty acid chain, the one after the colon denotes number of double bonds; the position of double bonds has not been determined the symbol x denotes unidentified components, 12:0 n-dodecanoic = lauric,

of Neisseria and Moraxellae

Fatty acid†‡												
15 0	x-4	16:1	16:0	3-OH 16 0	17 1	17-0	x 5	x-6	18 2	18:1	18 0	others
–	–	22	38	–	tr	–	tr	tr	3	10	3	–
–	–	29	30	–	tr	–	tr	1	1	14	2	6
tr	–	33	41	–	–	–	–	tr	tr	8	2	–
tr	–	25	37	–	–	–	–	tr	1	10	2	–
tr	tr	29	29	–	tr	tr	–	tr	1	20	2	5
2	–	21	28	4	1	1	–	tr	1	18	2	–
2	–	22	25	4	1	1	–	tr	1	19	2	–
2	–	25	39	3	tr	tr	–	tr	2	9	2	–
–	–	8	10	–	6	–	2	tr	5	55	2	–
–	1	13	12	–	4	1	3	tr	1	48	9	–
	tr	18	9	–	3	–	1	tr	4	49	3	–
–	1	13	13	–	4	–	1	tr	3	45	2	2
–	tr	9	8	–	4	–	2	tr	1	62	6	–
–	tr	7	7	–	3	–	3	tr	3	61	5	–
–	6	27	18	–	1	–	6	tr	3	22	[illegible]	5
–	3	4	4	–	2	–	24	tr	4	44	4	7
–	3	28	9	–	1	–	12	tr	1	33	2	1
	1	30	7	–	7	–	1	tr	4	37	2	2
–	1	9	9	–	4	–	8	tr	12	46	4	–
–	1	21	8	–	1	–	7	tr	5	45	2	3
tr	tr	23	8	–	2	–	2	tr	9	42	1	1
–	–	10	7	–	1	–	tr	–	–	59	12	–
	–	7	5	–	3	1	tr	tr	tr	68	8	–
–	–	12	14	–	1	tr	–	tr	30	25	5	–
–	–	15	15	–	tr	–	–	–	9	18	6	1
–	–	18	10	–	–	–	tr	tr	2	5	2	–
	–	14	10	–	–	tr	tr	tr	3	11	4	1
1	–	2	30	8	tr	1	–	–	tr	46	2	–

14:0 n-tetradecanoic = myristic, 15 0 n-pentadecanoic, 16 1 hexadecenoic, 16-0 n-hexadecanoic = palmitic, 17 1 heptadecenoic, 17-0 n-heptadecanoic, 18 2 octadecadienoic, 18 1 octadecenoic, 18-0 n-octadecanoic = stearic, 3-OH 12-0 3-hydroxy-dodecanoic 3-OH 14-0 3-hydroxy-tetradecanoic, and 3-OH 16:0 3-hydroxy-hexadecanoic acid.

‡ The fatty acid concentrations are given as percentage of the total. The symbol "tr" (trace) indicates that the acid contributes less than 0.5 per cent of the total.

TABLE 2. *Range of Two Fatty Acid Ratios for Groups§ of Neisseriae and Moraxellae*

Group/Species§	Σ C_{16} fatty acids† / Σ C_{18} fatty acids (scale 0–6)	Σ saturated fatty acids‡ / Σ unsaturated fatty acids (scale 0–2.5)
I "True neisseriae" (8)	├── ≈2 to >6 ──┤	├── ≈0.7 to ≈1.45 ──┤
II "False neisseriae" (6)	├ ≈0.2 to ≈0.7 ┤	├ ≈0.1 to ≈0.4 ┤
III "Classical moraxellae" (7)	├ ≈0.1 to ≈1.6 ┤	├ ≈0.1 to ≈0.45 ┤
IV *Moraxella osloensis* (2)	├ ≈0.2 to ≈0.5 ┤	├ ≈0.1 to ≈0.3 ┤
V *Moraxella phenylpyrouvica* (2)	├ ≈0.3 to ≈0.8 ┤	├ ≈0.35 to ≈0.55 ┤
VI *Moraxella kingae* (2)	├ ≈1.2 to ≈3 ┤	├ ≈1.95 to ≈2.5 ┤
VII *Moraxella urethralis* (1)	× (≈0.6)	× (≈0.8)

§ See text and Table 1 for the complete group, species and strain designations. Figures in parentheses indicate number of strains examined.

† The ratio between (16·0 + 16 1) and (18·0 + 18 1 + 18 2) the hydroxy fatty acids are not included. See Table 1

‡ The ratio between (12·0 + 14 0 + 15·0 + 16 0 + 17 0 + 18·0) and (16 1 + 17 1 + 18 1 + 18 2) the hydroxy fatty acids are not included. See Table 1

related, both lacking methyl ester groups. In partitioning experiments with acetonitrile/hexane (17) x-4 and x 5 were considerably less polar than normal fatty acid methyl esters.

Tables 1 and 2 (see also ref 17 Fig 4) show that the fatty acid patterns in group I "true neisseriae" and group II "false neisseriae" are strikingly different. The former is characterized by a high level of C fatty acids, the latter by less C_{16} and more C fatty acids. These two groups also differ in the ratio between saturated and unsaturated fatty acids which is higher in group I n Tetradecanoic acid (14:0) contributing 2–7 per cent to the total fatty acids in group I is only present in trace amounts in group II. Group III "classical moraxellae" shows a high resemblance to group II and is distinctly different from group I in all three characteristics. The compounds x-4 and x 5 appear to be characteristic of group II and group III The strains of *M osloensis* and *M phenylpyrouvica* are, generally rather similar to the groups II and III but each deviates in a few of the components. *M kingae* is characterized by unusually high amounts of 14·0 which is otherwise only present in small amounts in group I This predominance of 14·0 also contributes to a high level of saturated fatty acids in this species. A characteristic of the *M urethralis* strain is the lack of 3-OH 12·0 which is present in all other strains. The low ratio between 16 1 and 16·0 and the relatively high amount of 3-OH 16·0 are also distinctive of *M urethralis*

Numerical Analysis

Table 3 shows the calculated similarities in fatty acid composition for all pairs of strains. Cluster analysis based on these similarities shows (Fig 3) that "true neisseriae" form two closely associated homogeneous clusters (strains 1 2 3 4 and 5) and (strains 6, 7 and 8) *N elongata* having a high affinity to *N flavescens* is grouped with the true neisseriae" "False neisseriae" and moraxellae (with the exception of *M kingae* and *M urethralis*) form one big cluster *M kingae* and *M urethralis* are distinctly separated from the clusters mentioned, and from each other The fatty acid pattern of *M kingae* has some resemblance to those of the "true neisseriae" however

The group similarity matrix (Table 4) shows the intra and inter-group similarities. Both group I ("true neisseriae") and group

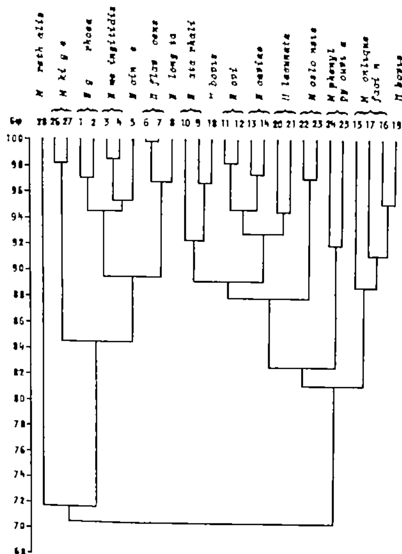


Fig 3 Phenogram of fatty acid contents of neisseriae and moraxellae obtained by unweighted pair group mean analysis according to *Sokal & Michener* (25) based on the primary similarity matrix (Table 3)

II ("false neisseriae") have relatively high homogeneity with mean similarities of 92.5 and 92.6. Group III ("classical moraxellae") and group (III+IV+V) (i.e. group III plus *M. osloensis* and *M. phenylpyrouvica*) are less homogeneous (mean similarities 87.1 and 82.2 respectively). Group (II+III) is more homogeneous than group III. The affinities between group II and group III and between the former and group (III+IV+V) are of the same order (87.0 and 87.1). This indicates a close association between "false neisseriae" and moraxellae. The fact that there is less homogeneity in group (III+IV+V) than in group III (or in II+III) means that *M. osloensis* and *M. phenylpyrouvica* implement an increased heterogeneity of the genus *Moraxella*. The group matrix shows that *M. kingae* in terms of fatty acid composition resembles the neisseriae of group I more than any other group of bacteria examined, but this affinity is relatively low. *M. urethralis* only has low proximities to the other entities.

TABLE 3 *Similarity Matrix of Fatty Acid Composition of Neisseriae and*

Species	Strain§	1	2	3	4	5	6	7	8	9	10	11	12
1 *N gonorrhoeae*	21319/70	×											
2 *N gonorrhoeae*	362/71	97	×										
3. *N meningitidis*	M1	93	94	×									
4 *N meningitidis*	B8152/66	85	95	99	×								
5 *N cinerea*	159/62	95	95	95	96	×							
6. *N flavescens*	13120	87	87	90	90	91	×						
7 *N flavescens*	8263	87	87	90	90	91	100	×					
8. *N elongata*	M2	89	88	93	93	90	97	97	×				
9 *N catarrhalis*	Ne 11	80	78	72	73	82	71	72	71	×			
10. *N catarrhalis*	13074/62	79	80	75	74	82	70	70	69	91	×		
11 *N ovis*	199/55	87	86	79	81	87	78	78	78	93	89	×	
12. *N ovis*	37/59	83	82	77	78	84	74	74	74	88	86	98	×
13 *N caviae*	14659	85	85	78	78	85	76	76	74	93	92	97	94
14 *N caviae*	10293	85	84	75	77	84	75	76	73	92	90	95	93
15. *M nonliq*	4663/62	66	65	68	67	74	64	64	64	83	88	81	80
16. *M nonliq*	7784	58	57	54	53	65	54	55	53	84	90	81	81
17 *M nonliq*	5067/66	68	69	69	67	74	63	63	64	84	89	83	83
18. *M bovis*	10900	79	79	74	74	82	73	73	73	97	93	89	86
19 *M bovis*	4	64	62	57	58	69	61	61	60	89	91	89	88
20 *M lacunata*	17967	77	76	73	73	81	72	72	71	89	90	93	90
21 *M lacunata*	7911	83	81	73	76	83	78	78	76	91	87	95	92
22. *M osloensis*	A1920	79	80	78	78	86	83	82	78	86	91	95	94
23 *M osloensis*	5873	73	74	70	70	81	78	77	70	82	89	91	89
24 *M phenylpyr*	2863	74	71	69	71	76	66	66	67	86	81	85	81
25 *M phenylpyr*	17958	86	81	80	82	88	82	83	83	91	89	90	86
26. *M kingae*	4177/66	83	83	90	91	86	79	79	83	61	64	71	68
27 *M kingae*	9076/70	86	85	90	91	86	78	79	81	67	66	74	71
28. *M urethralis*	WM6	68	68	70	67	72	84	83	80	58	62	68	66

See text for definition and explanation. § See Table 1 for the complete strain designations.

Reproducibility

N catarrhalis Ne 11 was harvested from nine batches of standard blood agar medium (16) of which three batches were prepared in one laboratory and six in another Three of the latter batches contained blood from the same human donor whereas the remainder differed with respect to blood donors. The similarity indices between pairs of fatty acid patterns were uniformly above 98. Also, the absence of blood in the agar had no significant influence on the GLC profile for this organism.

As most strains of "true neisseriae" were grown at 37 C (with initial CO supplement) and not at the standard temperature of 33 C (see *Materials and Methods*) the possible effect of this difference in growth conditions was studied in separate experiments. Two strains of this group (*N meningitidis* B8152/66 and *N elongata* M2) were grown under both conditions and the corresponding fatty acid compositions were determined. The fatty acid patterns at the two temperatures rendered similarity indices of 99.8 for each strain. Both strains disclosed a slightly lower ratio of saturated to unsaturated fatty acids when grown at 33 C. However in distinction from the "false neisseriae" and moraxellae the ratio was still in the range 1 1–1.3 (see Table 2)

DISCUSSION

The fatty acids of the genus *Neisseria* have been studied previously (18 20 22, 26) *Moss et al* (22) found that the two patho-

...*ierellae Strains Expressed by a Transformed Yule Correlation Coefficient**

13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
x															
97	x														
81	78	x													
83	80	88	x												
83	78	91	92	x											
89	87	85	83	86	x										
86	84	88	95	90	89	x									
93	94	86	87	86	87	88	x								
82	94	81	81	79	89	85	95	x							
93	93	73	73	79	83	74	86	86	x						
91	87	70	72	74	81	73	80	83	97	x					
79	76	72	69	72	87	81	74	80	76	76	x				
86	83	81	72	79	92	81	82	86	76	78	92	x			
68	64	56	44	63	66	46	62	64	61	54	66	73	x		
72	71	60	52	87	69	53	67	68	66	56	67	77	98	x	
67	61	5	50	55	58	50	60	61	68	67	53	53	63	63	x

genic species *N gonorrhoeae* and *N meningitidis* have essentially the same fatty acid patterns. *Lambert et al.* (18) demonstrated, in conformity with our findings, distinct fatty acid differences between the groups of neisseriae which correspond to our designations "true" and "false" In accordance with our previous findings (16) these authors observed that the fatty acid content was influenced by growth medium composition but that species specific results were obtained regardless of the media used. When our results differ quantitatively from those of *Moss et al.* and *Lambert et al.*, this may to some extent be due to differences in media and growth conditions. The marked divergence in contents of C–C fatty acids, however is most probably the consequence of differences in chemical procedures.

Generally the results of the present study are in accordance with previous findings on GLC finger prints" of the same strains of bacteria (9 11† 16) Thus, "true neisseriae" (*N gonorrhoeae N meningitidis N cinerea, N flavescens* and *N elongata*) and "false neisseriae" (*N catarrhalis N ovis* and *N caviae*) are both relatively homogeneous groups distinct from each other in terms of fatty acid composition One outstanding difference is the high content of C_1 fatty acids in the former group and of C_1 acids in the latter as reported also by others (18) Another significant dissimilarity is the ratio of saturated to unsaturated fatty acids this is

† Nearly identical f tty acid patterns were found for fimbriated (Sc-a) and nonfimbriated (N-a) variants of *Moraxella nonliquefaciens* NCTC 7784

rodshaped human parasites of these groups (*M nonliquefaciens M osloensis M phenylpyruvica M lacunata, M kingae M urethralis* and *N elongata*) have distinctive fatty acid patterns. If this proves true when a larger number of genetically allocated strains of each species are compared, the procedure could be utilized for the otherwise mostly cumbersome identification of these bacteria in the diagnostic laboratory

The mass spectral analyses were performed at the Institute of Clinical Biochemistry University of Oslo, Rikshospitalet, Oslo. The valuable assistance of Dr *E. Jellum* and Mr *P Helland* of that institute is gratefully acknowledged.

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ISOLATION AND PURIFICATION OF DEOXYRIBONUCLEIC ACID FROM MYCOBACTERIA

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An account is given of isolation of DNA from mycobacteria. Mechanical disruption was carried out in a French pressure cell press, followed by isolation of DNA by means of Marmur's method. This DNA contained considerable amounts of polysaccharides which, however could be removed by precipitation with cetyltrimethylammonium bromide (CTAB). The degree of purity obtained and the GC contents are given.

In order to be able to demonstrate minor differences in the DNA from various bacterial species or strains, isolation of purified DNA is essential.

DNA from mycobacteria has been extracted both after mechanical disruption (*Chargaff & Saidel* 1949, *Tsukamura et al.* 1960, *Venner* 1963, *Sellers & Tokunaga* 1966, *Tarnok et al.* 1967, *Tewfik & Bradley* 1967, *Slosárek* 1970, *Kraus et al.* 1973) and after enzymatic lysis of the cells (*Wayne & Gross* 1968, *Mizuguchi & Tokunaga* 1970, *Slosárek* 1973, *Bradley* 1973). In principle, enzymatic treatment of the intact bacteria would be preferable if endogenous DNase activity could be inhibited, because a risk of infection of personnel might be involved in some of the mechanical methods and the danger of degradation of the DNA resulting in a low molecular weight of the final product. A high molecular weight is of significance in, for instance, transformation experiments.

It was difficult for the author to obtain alcohol precipitation of DNA from mycobacteria in attempts at lysing the bacteria with lysozyme or pronase. In order to find a reproducible method for isolation of DNA, the French pressure cell press was used and subsequent purification by Marmur's method (*Marmur* 1961). However the DNA obtained was found to contain large amounts of high-molecular polysaccharides which influence measurements at 260 nm in the spectrophotometer and which possibly affect hybridization experiments (*De Ley et al.* 1970). After several trials, it was decided to use precipitation by cetyltrimethylammonium bromide (CTAB) according to a modification by Hill et al. of Darby's method (*Hill et al.* 1972).

MATERIAL AND METHODS

Bacteria. The strains used were the Danish strain of *M. bovis BCG* and *M. smegmatis* (ATCC 607).

Isolation of DNA. The bacteria were grown on flasks each containing 180 ml Sauton medium, the BCG strain for about 23 days and the *M. smegmatis* strain for about 5 days. Filtration was made by means of a Büchner funnel attached to a suction device. The bacteria were washed with saline-EDTA

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(0.15 M NaCl, 0.1 M ethylenediaminetetraacetate, pH 8) and left at 4 C until the next day when a quantity of saline-EDTA, sufficient to obtain a consistency of a thick paste, was added.

The bacteria were passed through the French pressure cell press (American Instrument Company) at a pressure of 14000 psi. A further amount of saline-EDTA and a 0.1 volume of 5 per cent sodium deoxycholate was added immediately afterwards. The suspension was placed in a waterbath at 56 C for 90 minutes and shaken occasionally

Isolation of DNA was mad by the method described by *Marmur* (1961) but with two minor modifications. After cooling to room temperature, 5 M sodium perchlorate solution was added to a final concentration of 1 M. Shaking was made with an equal olume of chloroform-iso-amyl alcohol, 24 1 (/) for 30 minutes. The suspension was centrifuged at 8000 rev/min for 5 minutes and the supernatant aqueous phase removed by pipette. Treatment with chloroform-iso-amyl alcohol was repeated, using shaking for 15 minutes instead of 30 minutes, until the protein layer had almost disappeared (about 3–4 times) The DNA was then precipitated with two volumes of 95 per cent ethyl alcohol, twisted up on a glass rod, and dissolved in SSC (0.15 M NaCl, 0 015 M trisodium citrate, pH 7.0) corresponding to 0.5 to 0.75 of the volume of the supernatant. An amount of 50 µg RNase per ml, from a solution of 1000 µg/ml in 0.15 M NaCl, pH 5 was added. This solution had been placed at 92° C for 10 minutes in order to inactivate traces of DNase. Incubation was made at 37 C for 30 minutes, after which shaking with one volume of chloroform-iso-amyl alcohol, centrifugation and removal of supernatant, were repeated as many times as necessary to eliminate the intermediate protein layer (generally about three times) The DNA was precipitated again with tw volumes of 95 per cent ethyl alcohol and dissolved in 9 ml 0.1 × SSC. An amount of 1 ml acetate EDTA (3 M sodium acetate, 0.001 M ethylenediaminetetraacetate, pH 7) was added to the solution followed by dropwise addition of 5.4 ml (possibly more) isopropyl alcohol during rapid mechanical stirring with a glass rod. The DNA on the glass rod was washed in alcohol in concentrations increasing from 70 to 95 per cent, dissolved in a suitable amount of SSC and stored t 4 C. A few drops of chloroform may be added.

Purification of DNA for polysaccharides The modification by Hill et al. of Darby method with precipitation with CTAB (*Hill et al.* 197) was used. The DNA was dialysed against and diluted with 0.4 M NaCl to a concentration of about 500 µg/ml. 2 ml of CTAB at a concentration of 5 per cent in 0.4 M NaCl was added per 5 ml of DNA solution. After 15 minutes at room temperature, little more (about 5 drops) of the CTAB solution was added in order to ensure complete precipitation. The precipitated DNA was washed twice in 0 4 M N Cl and then dissolved in 1 M NaCl. The solution was shaken with one volume of chloroform-iso-amyl alcohol 24 1 (v/v) for 15 minutes, centrifuged at 8000 rev/min for 5 minutes, and the aqueous phase was removed. This treatment must be repeated if an intermediate layer is found. The DNA was then precipitated with 3 volumes of 95 per cent alcohol dissolved in SSC, and dialysed against SSC.

M asurements of the final DNA The concentration was determined both by measuring the optical density at 260 nm in the spectrophotometer and by diphenylamine according to Burton (*B rton* 1968) using 2'-deoxyadenosine-5 -monophosphoric acid as standard, corresponding to from 10 to 150 µg DNA/ml.

The increase in the optical density of DNA at 260 nm was measured after denaturation with KOH. To the solution was added 3 M KOH to a final concentration of KOH of 0.3 M and determination was carried out after storage at room temperature for 15 minutes. The degree of dilution was taken into consideration in the calculation of hyperchromicity

As indication of the purity of the DNA from protein and polysaccharide, the optical density ratios 260.280 nm and 260.230 nm were measured (*Marmur* 1961)

The content of polysaccharide was determined according to the anthrone method (*Umbreit & Burris* 1964) The standard concentrations used ranged from 30 to 120 µg glucose in 3 ml distilled water Distilled water was used as control. 200 mg anthrone was dissolved in 100 ml 95 per cent H_2SO 3 ml of standard or sample was mixed rapidly with 6 ml reagent, boiled for 3 minutes in waterbath, cooled, and read at 620 nm in the spectrophotometer

The content of protein was determined by Folin-Ciocalteu reagent (*Bailey* 1962) The standard used was bovine albumin (Armour) in amounts of from 10 to 150 µg in 0 2 ml distilled water

Reagents

A. 2 g NaCO dissolved in 100 ml 0 1 n NaOH
B 1 g sodium citrate and 0.5 g CuSO 5 H_2O dissolved in 100 ml distilled water
C. 1 ml of B mixed with 50 ml of A.
D 10 ml Folin-Ciocalteu reagent mixed with 9 4 ml distilled water

0 ml standard solution or sample was mixed with 2 ml of C. The mixture was allowed to stand t room temperature for 10 minutes. 0.2 ml of D was mixed in rapidly and the mixture was read after a time interval of ½–2 hours. Determination was made by spectrophotometer t 750 nm.

RNA determination was not performed because

TABLE 1 *Measurements of DNA Isolated from* M smegmatis *and* M. bovis, *BCG*

	DNA from *M smegmatis*			DNA from *M bovis, BCG*	
	Unpurified	Unpurified	Purified	Unpurified	Purified
Optical density ratio 260 280 nm	1.91	1.88	1.92	1.86	1.94
Optical density ratio 260 230 nm	–	2.08	2.32	–	2.25
Hyperchromicity (in %) at 260 nm after denaturation with KOH	23.2	26	28.1	25 1	34 1
mg polysaccharide per mg DNA	0.867	0.961	0.020	3 75	0.096
Protein content (in %)	0.8	1 4	0.17	1..	0.6
T_m in C (0.1 × SSC)	–	82.2	82.2	81	81
GC content (in %)	–	69 05	69.05	66.1	66.1

the polysaccharides of the mycobacteria contain pentoses which give positiv reactions if the orcinol and cysteine hydrochloride methods are used.

Determination of GC content by means of thermal denaturation was performed with Zeiss spectrophotometer PMQ III using quartz cells with cm path length and a magnetic stirrer embedded in glass. A thermistor connected with a galvanometer showing the temperature with an accuracy of 0.1 C was inserted into the sample itself The temperature-controlled cell holder was connected with a thermostatically controlled waterbath circulating at a rate of 8 l per minute. In addition to water cooling of the sample changer special filter had to be fitted to protect the photomultiplier against heat from the cell holder The salt concentration was reduced in order to keep the temperature below 100 C. Instead of SSC, 0.1 × SSC was used, and for determination of the GC content, the form la GC = (T_m – 53 9) 2.44 was applied, as described by *Mandel & Marmur* (1968) The DNA concentration in the cells was about 20 µg/ml, corresponding to an extinction of about 0.8 at a path length of 2 cm. Correction was made for fluid expansion at increasing temperatures. After reading at 25 C, the temperature was raised rapidly to 70 C. After that temperature had been achieved, readings were made t increments of 2° C and, d ring the denaturation process itself at increments of 1 C, after which larger increments were again used Temperature equilibrium in the sample cell was waited before each reading (approximately 10 minutes for an interval of 1 C)

RESULTS

After extraction by means of Marmur's method (*Marmur* 1961) the yield of DNA was about 1 mg per 1 g bacteria (wet weight) The subsequent purification for polysaccharides by CTAB resulted in a further loss of 14 per cent of *M smegmatis* DNA and 57 per cent of BCG DNA, but more than 95 per cent of the polysaccharides had been removed. This resulted in a better concordance between the determinations of the amounts of DNA by spectrophotometer and by diphenylamine The values obtained by spectrophotometer were too high before purification on account of the absorption of light by the polysaccharides at 260 nm. The other determinations are shown in Table 1 Purification of DNA from *M smegmatis* was performed with a mixture of two different preparations, both of which are indicated.

DISCUSSION

By the method used in the present study it is possible to isolate DNA from both rapidly and slowly growing mycobacteria with a satisfactory degree of purity The DNA has a low molecular weight (1 to 2 × 10^6 daltons) (unpublished results) This is probably due to the effect of mechanical disruption in the pressure cell press and the repeated extraction with chloroform-iso-amyl alcohol Enzymatic methods for lysis of the bacteria presumably result in DNA with a higher molecular weight if endogenous degradation can be avoided during the enzymatic process. Repeated experiments with lysozyme or pronase per

formed in this study resulted in DNA which was presumably degraded and therefore could not be isolated by extraction according to *Marmur* (1961)

It has been shown in unpublished experiments that removal of polysaccharides by chromatography with DEAE Sephadex A50 was effective but unpredictable since the DNA was degraded in the majority of instances. Purification with CTAB (*Hill et al.* 1972) was less time-consuming and better reproducible.

The fact that it is necessary to remove the polysaccharides from the DNA has been shown clearly in isolating DNA from *M gastri* Before the CTAB treatment, the DNA contained 28.44 mg polysaccharides per mg DNA, and the diphenylamine determination showed that there was only half as much DNA in the solution as otherwise measured by spectrophotometer at 260 nm. The optical density ratio 260:280 nm rose after purification from 1.59 to 1.87 and the ratio 260:230 nm from 1.07 to 2.03 The hyperchromicity after denaturation with KOH changed from 9.3 per cent to 26.2 per cent.

As shown in the table, the polysaccharides had no effect in determinations of the GC content by means of thermal denaturation. This is in agreement with *Hill et al.* (1972)

It was found by *Hill et al.* (1972) and also by the present authors using BCG that there was an increase in the ratio 260:280 nm after CTAB treatment of DNA. However in the present study no intermediate layer was observed after centrifugation following shaking with chloroform-iso-amyl alcohol. This is probably due to the fact that Hill's DNA contained more protein at that stage than the DNA isolated in this study which had been treated by Marmur's method, possibly because of the dissociation of protein from DNA by the sodium perchlorate.

The GC content is somewhat higher in this study than that which according to previously reported values has been found to apply to the same bacterial strains. This may be due to variations in technique. For instance, different buffers were used and the T_m value is dependent on the ionic strength of the solvent (*Mandel & Marmur* 1968) Furthermore in the present study the temperature was measured directly in the cell containing the DNA solution, and this was lower than the temperature in the waterbath used for heating the cell holder This indicates how important it is to measure the temperature in the sample itself.

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GAS CHROMATOGRAPHY OF BACTERIAL WHOLE CELL METHANOLYSATES

VI Fatty Acid Composition of Strains within Micrococcaceae

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Strains of the genera *Micrococcus* and *Staphylococcus* were examined for fatty acid content by gas-liquid chromatography and the data analysed by a numerical procedure. The fatty acids rendered a clear-cut distinction between the genera. Characteristic patterns were found for *M roseus* and *M mucilaginosus*. *M luteus* on the other hand, appeared more heterogeneous. The fatty acid patterns of *S aureus* and *S epidermidis* were overlapping *S epidermidis* being split into two clusters. *S saprophyticus* appeared in a subcluster together with three coagulase negative staphylococci.

Classification of Gram-positive cocci by traditional procedures has led to considerable confusion. Recent improvements have been achieved by the introduction of procedures for determination of DNA homology and biochemical methods for the analysis of cell wall composition. For instance, the mole per cent guanine + cytosine (per cent (G + C)) of DNA separates the *Micrococcaceae* at the genus level (17) and enabled a re-allocation (21) of strains classified erroneously by the procedure of anaerobic glucose breakdown suggested by the Subcommittee on Taxonomy of Staphylococci and Micrococci (28) Similarly peptidoglycan and teichoic acid patterns allow subdivision of coagulase negative staphylococci which, although lumped together under the designation *Staphylococcus epidermidis* in the 7th edition of Bergey's Manual, encloses several different biotypes (24) A combination of peptidoglycan analysis and genetic transformation has contributed to a redefinition of *Micrococcus luteus* (18, 19 22, 23)

The purpose of the present investigation was to extend the biochemical characterization of bacteria within the family *Micrococcaceae* The emphasis is here on analysis of fatty acids by gas-liquid chromatography (GLC) and how fatty acid patterns coincide with preconceived classification of a set of carefully chosen bacterial strains.

MATERIALS AND METHODS

Bacterial Strains

A few well-defined species within the *Micrococcaceae* were considered. The individual strains are listed in Table 1 The per cent (G+C) of all selected strains was known as were also the peptidoglycan and teichoic acid patterns of most strains (Table 2) With the exception of some of the *M mucilaginosus* isolates, all strains were obtained from Dr. *M Kocur* Czechoslovak Collection of Microorganisms (CCM) Brno, Czechoslovakia. Most of the *M mucilaginosus* strains were described in

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TABLE 1 List of Strains Examined

Identification number	Species	Strain designation CCM	ATCC	NCTC	Other	Previous name[a]
1[1]	*Micrococcus luteus*	210	400 = 10240a	2678	NCIB 10419	Entered ATCC as *M. flavus*
2.	*Micrococcus luteus*	337	382		AMNH 208	*Sarcina lutea*
3.	*Micrococcus luteus*	732	10240	7743	NCIB 8166, FDA 16	*M. flavus*
4	*Micrococcus luteus*	1335				Entered CCM as *M. lysodeikticus*
5.	*Micrococcus luteus*	2266		7563		*Staphylococcus afermentans*
6.[2]	*Micrococcus* sp.	810	398	8512	NCIB 8165, IFO 3763 IAM 1097	Entered ATCC and CCM as *M. luteus*
7	*Micrococcus varians*	268				
8.	*Micrococcus mucilaginosus*	2417	25296	10663		Entered CCM as *Sarcina flava*; *M. lactis*
9	*Micrococcus mucilaginosus*	2392				Also referred to previously as *Staphylococcus salivarius*
10.	*Micrococcus mucilaginosus*	2393				
11	*Micrococcus mucilaginosus*	2486				
12.	*Micrococcus mucilaginosus*				4239/68	
13	*Micrococcus roseus*	679	186	7523	IAM 1365 IFO 3768	*M. rubens*
14	*Micrococcus roseus*	337	177	7512		
15	*Micrococcus roseus*	837	416	7518		
16.	*Micrococcus roseus*	2179	14396			*M. infimus*
17	*Micrococcus roseus*	2237	144	7511		*Staphylococcus roseus*
18.[4]	*Staphylococcus aureus*	885	12600	8532		
19	*Staphylococcus aureus*	1484		4163		
20.	*Staphylococcus aureus*	2022	6538	7447	NCIB 8625	
21	*Staphylococcus aureus*	2301		4136		
22.	*Staphylococcus aureus*	2351	10832	7121		
23.[7]	*Staphylococcus epidermidis*	2124	14990			
24	*Staphylococcus epidermidis*	50		9865		*M. candidus gabriella*[11]
25.	*Staphylococcus epidermidis*	446				
26	*Staphylococcus epidermidis*	2210		189		*Staphylococcus lactis*
27	*Staphylococcus epidermidis*	2340		7944		*Staphylococcus lactis*
28.	*Staphylococcus* sp.	2429				*Staphylococcus lactis*
29	*Staphylococcus saprophyticus*	883	15305	7292	NCIB 8711	

[1] Proposed neotype strain of *M. luteus* (18)

One time neotype strain of *M. luteus* (4). After redefinition of species and designation of new neotype (18, 23), this strain no longer can be considered part of *M. luteus*

Proposed neotype strain of *M. varians* (19)

Proposed neotype strain of *M. mucilaginosus* (1)

Proposed neotype strain of *M. roseus* (18)

Proposed neotype strain of *S. aureus* (6)

Proposed neotype strain of *S. epidermidis* (9)

Proposed neotype strain of *S. saprophyticus* (24)

See *Kocur et al.* (17, 18), *Kocur & Martinec* (19), and *Schleifer et al.* (23)

[10] *Gordon* (8)

Suggested as separate species (5)

Nomenclature of *Silvestri & Hill* (26)

TABLE 2. P ptidoglycan Composition Teichoic Acid and Per Cent Guanine + Cytosine Content f DNA (per cent (G+C)) f the St ains Studied

Identification number	Species	Strain designation CCM	Peptidoglycan*	Teichoic acid	Per cent (G+C)§
1	*Micrococcus luteus*	210	L-Lys-peptide subunit		72.8
2	*Micrococcus luteus*	337	L-Lys-peptide subunit		72.0
3.	*Micrococcus luteus*	732	L-Lys-peptide subunit		65.0
4	*Micrococcus luteus*	1335	L-Lys-peptide subunit		72.0
5.	*Micrococcus luteus*	2268	L-Lys-peptide subunit		72.8
6.	*Micrococcus* sp.	810	L-Lys-Gly-L-Glu		66.0; 66.3
7	*Micrococcus varians*	268	L-Lys-L-Ala_{3-4}		71.8 72.0
8.	*Micrococcus mucilaginosus*	2417	L-Lys-L-Ala		59.0
9	*Micrococcus mucilaginosus*	2392	L-Lys-L-Ser(L-Ala)		58.3 60.0
10.	*Micrococcus mucilaginosus*	2393	L-Lys-L-Ser(L-Ala)		55.9
11	*Micrococcus mucilaginosus*	2486	L-Lys-L-Ala		59 0
12.	*Micrococcus mucilaginosus*	4239/68			59.0
13.	*Micrococcus roseus*	679	L-Lys-L-Ala_{3-4}		69.0 68.0
14	*Micrococcus roseus*	557	L-Lys-L-Ala_3		72.8
15	*Micrococcus roseus*	837	L-Lys-L-Ala_{3-4}		69.7 70.0
16.	*Micrococcus roseus*	2179	L-Lys-L-Ala_{3-}		72.8 71.0
17	*Micrococcus roseus*	2237	L-Lys-L-Ala_{3-4}	Ribitol	73.0
18.	*Staphylococcus aureus*	885	L-Lys-Gly_{5-6}		31.2
19	*St phylococcus aureus*	1484			31.2
20.	*Staphylococcus aureus*	2022			35 7 37 7
21	*Staphylococcus aureus*	2301			32.8
22.	*Staphylococcus aureus*	2351			33.4
23	*Staphylococcus epidermidis*	2124	L-Lys-Gly_{4-5} L-$Ser_{0.5-1}$	Glycerol	34.6
24	*St phylococcus pidermidis*	50	L-Lys-Gly_{4-5} L-$Ser_{0.5-1.5}$	Glycerol	30.0
25.	*Staphylococcus epidermidis*	2446	L-Lys-Gly_{4-5} L-Ser	Glycerol	33.0
26	*Staphylococcus epidermidis*	2210	L-Lys-Gly_5	Ribitol + Glycerol	32.0
27	*St phylococcus epidermidis*	2340	L-Lys-Gly_5	Glycerol	36.5
28.	*Staphylococcus* sp.	2429			30.9; 33 0
29	*Staphylococcus saprophyticus*	883	L-Lys-Gly_{4-5} L-$Ser_{0.5-1.5}$	Ribitol	31.6

* Data from *Schleifer & Kandler* (22) and *Schleifer & Kocur* (24)

§ Data from *Kocur et al.* (17)

Species	Strain designation§ CCM	Fatty acid†‡ 14:0-i	14:0	15:0-i	15:0-a	15:0	16:0-i	16:0	16:1
1 *M luteus*	210	2‡	1	2	48	tr	12	5	2
2 *M luteus*	337	3	1	8	62	tr	8	2	1
3 *M luteus*	732	5	1	28	54	tr	1	3	–
4 *M luteus*	1335	3	3	22	52	2	4	4	1
5 *M l teus*	2266	4	2	19	33	1	13	4	tr
6 *M* sp.	810	tr	tr	5	60	tr	3	4	tr
7 *M varians*	268	3	1	13	41	tr	4	6	tr
8 *M mucilagi osus*	2417	8	2	2	38	tr	20	4	–
9 *M mucilaginosus*	2392	5	3	2	27	12	18	15	–
10 *M m cilaginosus*	2393	5	2	2	26	2	22	17	–
11 *M mucilaginosus*	2486	8	2	2	43	2	18	12	–
12 *M mucilaginosus*	4239/68	12	3	3	40	2	12	8	–
13 *M roseus*	679	1	2	7	53	tr	2	6	2
14 *M roseus*	557	1	3	7	64	tr	1	8	1
15 *M roseus*	837	1	2	14	37	1	3	4	7
16 *M roseus*	2179	1	2	17	57	tr	2	3	4
17 *M roseus*	2257	1	3	12	65	tr	1	4	4
18 *S aureus*	885	2	1	4	39	–	1	3	–
19 *S aureus*	1484	tr	tr	4	39	–	1	2	–
20 *S aureus*	2022	2	2	3	43	–	3	1	–
21 *S aureus*	2301	2	1	7	39	–	2	3	–
22 *S aureus*	2351	1	tr	4	42	–	2	3	–
23 *S pidermidis*	2124	2	tr	10	28	–	2	2	–
24 *S epidermidis*	50	2	tr	6	34	–	2	1	–
25 *S epidermidis*	2446	1	tr	5	34	–	1	1	–
26 *S pidermidis*	2210	2	1	10	56	–	1	3	–
27 *S epidermidis*	2340	1	tr	13	43	–	2	6	–
28 *S* sp.	2429	tr	tr	8	55	–	1	4	
29 *S sap phyticus*	885	tr	tr	10	46	–	tr	2	–

See text and ref 12 for experimental details.

§ See text and Table 1 for the complete strain designations.

† Key to the fatty acid designations the *figure before colon indicates number of carbon atoms* in the fatty acid chain, the one after colon denotes number of double bonds the positions of double bonds ha e not been determined i and -a indicate *iso* and *anteiso* position of the methyl branch the symbol x denotes unidentified component, 14 0-i 12-methyl tridecanoic, 14-0 n-tetradecanoic = myristic 15-0-i 13-methyl tetradecanoic, 15:0-a 12-methyl tetradecanoic, 15 0 n pentadecanoic, 16-0-i

conjunction with the rediscovery of the species (2) All strains were maintained in the lyophilized state and subcultivated only shortly before preparation for GLC.

Growth Conditions and Harvesting

The details of cultivation on blood agar (20 hours at 33 C in a humid atmosphere) and of harvesting ha e been described earlier (11)

Chemical Methods

Depolymerization and methyl ester formation took place in one step by methanolysis (2 N HCl in anhydrous methanol) as described previously (12) The fatty acid esters liberated were extracted directly from the reaction mixture by hexane (3 × 1 ml) (Merck p.a., redistilled) After one washing with 0.5 ml of distilled water the extract was concentrated to dryness by a stream of pure nitrogen. The material was then dissolved in 100 µl hexane and stored in sealed capillary tubes at

Fatty acid†‡												
17 0-i	17 0-a	17-0	18-0-i	**	18-0	18 1	19 0-i	19-0-a	19-0	20-0-i	20-0	other
–	15	tr	tr	–	2	3	–	–	1	tr	–	6
–	2	tr	–	–	1	3	–	–	tr	tr	–	9
–	1	tr	–	–	2	1	–	–	tr	–	–	3
1	–	1	–	–	2	2	–	–	tr	–	–	2
1	12	tr	–	–	1	1	–	–	tr	tr	–	9
1	16	–	–	–	1	1	–	–	tr	–	–	8
–	16	–	tr	–	2	3	–	–	1	3	–	7
–	5	–	–	–	2	3	–	–	1	–	–	5
–	8	tr	–	–	2	3	–	–	1	–	–	3
–	10	tr	–	–	2	2	–	–	1	–	–	9
–	4	tr	–	–	3	3	–	–	1	–	–	2
–	2	–	–	–	3	4	–	–	2	–	–	8
3	12	1	1	1	2	2	–	–	4	–	–	1
tr	5	1	2	1	2	2	–	–	1	–	–	1
–	6	4	5	7	1	2	–	–	3	–	–	2
–	2	1	1	1	1	2	–	–	3	–	–	2
	3	1	2	1	2	1	–	–	tr	–	–	1
1	7	tr	1	–	15	5	1	5	4	tr	9	2
4	22	tr	1	–	8	3	2	8	tr	–	3	2
3	16	–	3	–	7	1	1	5	tr	1	8	2
3	9	tr	2	–	12	2	tr	2	2	tr	10	4
2	12	tr	2	–	12	2	tr	2	1	tr	10	4
8	10	tr	2	–	13	1	4	3	2	tr	10	2
6	12	tr	3	–	12	1	4	5	1	tr	9	2
6	17	tr	2	–	8	1	6	9	1	tr	6	1
3	9	tr	tr	–	4	1	1		1	–	5	1
3	9	1	tr	–	9	4	tr	1	3	–	2	1
4	13	tr	tr	–	5	2	1	1	1	–	3	1
8	17	tr	tr	–	4	1	3	2	1	–	4	1

14-methyl pentadecanoic, 16 0 n-hexadecanoic = palmitic, 16 1 hexadecenoic, 17 0-i 15-methyl hexadecanoic, 17 0-a 14-methyl hexadecanoic, 17 0 n-heptadecanoic, 18 0-i 16-methyl heptadecanoic, 18:0 n-octadecanoic, 18 1 octadecenoic, 19:0-i 17-methyl octadecanoic, 19:0-a 16-methyl octadecanoic, 19-0 n-nonadecanoic, 20 0-i 18-methyl nonadecanoic, and 20 0 n-eicosanoic acid.

** Probably admixed with unknown minor component(s)

‡ The fatty acid concentrations are given as percentage of the total. The symbol "tr" (trace) indicates that the acid contributes less than 0.5 per cent of the total

20° C until injection on the (glass) column of the gas chromatograph.

Gas-liquid chromatography and identification and quantitation of fatty acids were carried out as described previously (12, 13) The iso- and anteiso-isomers of the branched acid methyl esters were sufficiently separated on the 10 per cent EGA column (12) isothermally at 160 C, to allow quantitation. Discrimination between normal, iso- and anteiso-fatty acid methyl esters was achieved by comparison with selected standards on two columns, 10 per cent W982 and 10 per cent EGA, and by mass spectrometric analysis as previously described (12, 13)

Procedures of Numerical Analysis

The fatty acid contents, expressed as percentage of total fatty acids, were evaluated by a procedure of numerical analysis as previously described (15) The transformed Yule correlation coefficient was used as similarity index (3) As

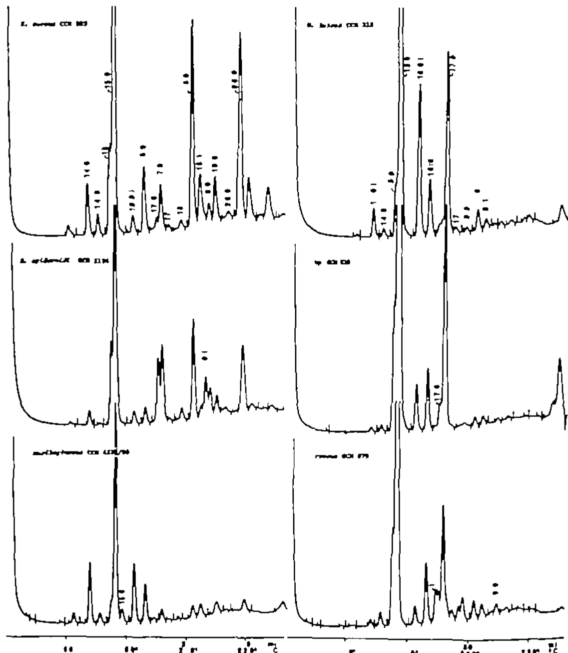


Fig. 1. Representative fatty acid profiles of Micrococcus *and* Staphylococcus *species.* Column 10 per cent EGA on Gas-Chrom Q, 200 × 0.2 cm glass (in dual mode). Temperature programmed 2° C/min from 140 C to 210 C. Flow rate of carrier gas 30 ml/min. Further chromatographic details are given in ref. 12. For fatty acid symbols see Table 3

the initial step, the data matrix (Table 3) was transformed by the formula $y = \ln (x + 1)$ Trace quantities (tr) were all given the value 0.2 per cent. The logarithmically transformed matrix was used for calculation of the affinity coefficients in the similarity matrix of primary elements (Table 4) Then followed clustering by the unweighted pair group cluster analysis (27)

RESULTS

Fatty Acid Composition

The relative fatty acid contents of each strain are shown in Table 3 Examples of elution profiles appear in Fig 1 The structure of one component, x, could not be fully elucidated by the procedure employed since it was not sufficiently separated from other peaks, the mass spectrometric examination was not conclusive. Selective extractions (12) however showed this component to be highly unpolar indicating a hydrocarbon structure.

The difference between the genera *Micrococcus* and *Staphylococcus* is pronounced. Particularly distinctive are the absence in *Micrococcus* and presence in *Staphylococcus* of a) the methyl branched C_{19} fatty acid isomers (19:0-i, 19:0-a) and b) the n-eicosanoic acid (20:0) (abbreviations explained in footnote to Table 3) Similarly 15:0 is present in *Micrococcus* and absent in *Staphylococcus*

The subdivision into species from fatty acid patterns appears relatively clear-cut within the genus *Micrococcus* Thus, *M mucilaginosus* lacks the 16:1 acid present in all other micrococcal species (except one strain of *M luteus*) It also contains high concentrations of 14:0-i and 16:0 acids. *M roseus* is the only species containing the structurally not fully elucidated component x. It is also the only *Micrococcus* species with more than trace amounts of the 15-methyl heptadecanoic (18:0-i) acid.

The intra-species heterogeneity of the fatty acid composition is most striking among the *M luteus* strains. For instance, the 17:0-a acid is entirely absent in one strain, two strains have 1–2 per cent, and two strains have the relatively high content of 12–15 per cent.

A most important observation is that the fatty acid patterns of *S aureus* and *S epidermidis* apparently are indistinguishable.

Numerical Analysis

Similarity Matrix of Primary Elements As shown in Table 4 the intra-species similarity coefficients are highest among strains within the species *M mucilaginosus* (88 to 98) *S aureus* (86 to 98) and *S epidermidis* (81 to 99) Within each of the species *M roseus* and *M luteus* the inter-strain similarities are a little lower and show more variation. Thus, the indices within *M roseus* range from 75 to 96 (only one value below 80) The relationships between strains belonging to different species are represented by distinctly lower indices for most pairs. However the similarity coefficients between *S aureus* and *S epidermidis* strains are generally indistinguishable from intra-species coefficients of each entity

Species Similarity Matrix Table 5 recording the preconceived species or groups, shows the matrix of mean transformed Yule correlation coefficients, mean deviations from the mean and standard deviations. Again it is seen that *M mucilaginosus* with a mean similarity of 94.3 has the highest intra-group similarity Staphylococcal species are next in homogeneity with 91–92 The mean similarity is lowest for *M luteus* with about 86. The species variation is seen to be least pronounced in *M mucilaginosus* and *S aureus* (mean deviation from the mean of only 2.6 and 2.9 per cent) and most marked in *M roseus* and *M luteus* (about 5 per cent) Within genus *Micrococcus* the inter-species similarities range from 67 to 86. *M roseus* exhibits slightly lower coefficients of similarity to the other micrococci, but still they are far above the indices for its similarity to staphylococci. In the genus *Staphylococcus* the inter-species similarities are high (90 between *S aureus* and *S epidermidis*)

Phenogram. In Fig 2 is shown the pheno-

ty And Composition of Micrococcus and
*Yule Correlation Coefficient**

14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
x															
87	x														
93	91	x													
96	88	92	x												
70	53	65	64	x											
64	45	53	59	88	x										
63	47	56	60	86	90	x									
74	58	68	68	95	88	90	x								
73	57	66	67	94	91	93	98	x							
83	47	58	58	89	92	92	91	93	x						
62	46	56	58	89	94	95	90	92	99	x					
61	45	54	56	88	96	93	86	89	97	99	x				
71	52	65	66	92	88	86	94	91	92	90	90	x			
76	59	71	69	88	84	77	92	91	87	83	81	90	x		
73	55	66	66	91	93	85	94	93	93	90	91	95	95	x	
63	49	58	59	88	94	84	87	88	94	92	95	94	88	97	x

It appears from the limited number of strains examined that the seven fatty acids 15:0, 16:1, 18:1 1, 19:0-I, 19:0-a, 20:0-i, and 20:0 and the unidentified component x have particular significance for genus and species differentiation within the *Micrococcaceae* (Table 3). As key components for distinction between the genera *Micrococcus* and *Staphylococcus*, the acids 19:0-i, 19:0-a, 15:0 and 20:0 can be considered. Each of these substances is present in only one of the genera. In species differentiation of micrococci, the 16:1 fatty acid and the x component appear particularly valuable. Thus, the absence of the former characterizes *M. mucilaginosus* and the presence of the latter is unique for *M. roseus*. The other fatty acids are not qualitatively specific alone, but quantitative species differences and particular combinations of peak sizes are apparent.

The interpretation of fatty acid composition for taxonomic purposes can be performed by consideration of key fatty acids as indicated. However, when comparisons have to be based mainly on quantitative variations of numerous chemical components in a large number of strains, the interpretation of GLC profiles easily becomes unwieldy by subjective means. Numerical analysis of the fatty acid concentrations is therefore of considerable significance. In this procedure* the trans-

* The choice of numerical procedure followed investigations of various analytical methods (unpublished results).

TABLE 3. *Matrix of Affinity and Homogeneity of Species of Micrococcus and Staphylococcus Based on their Fatty Acid Composition as Expressed by the Transformed Yule Correlation Coefficient*

Species	Variable	*M. luteus*-group	*M. luteus*	*M.* sp. CCM 810	*M. varians* CCM 268	*M. mucilaginosus*	*M. roseus*	*S. aureus*	*S. epidermidis*
M. luteus-group	S_T	83.7							
	Δ	5.4							
	σ	7.3							
M. luteus	S_T	–	86.4						
	Δ	–	4.8						
	σ	–	6.4						
M. sp. CCM 810	S_T	84.9	84.5	100					
	Δ	6.0	6.9	–					
	σ	8.6	9.6	–					
M. varians CCM 268	S_T	85.5	85.3	86.7	100				
	Δ	5.6	6.2	–	–				
	σ	8.0	8.9	–	–				
M. mucilaginosus	S_T	85.3	82.5	79.0	83.9	94.3			
	Δ	5.6	6.2	2.9	2.4	2.6			
	σ	8.9	7.6	3.9	3.1	3.2			
M. roseus	S_T	77.9	78.2	80.5	73.3	67.0	88.2		
	Δ	6.4	6.3	6.9	5.8	5.8	4.5		
	σ	8.1	7.6	9.5	8.5	8.5	6.1		
S. aureus	S_T	69.5	67.2	78.9	71.6	63.4	63.0	91.5	
	Δ	5.5	4.5	2.6	5.5	6.2	6.3	2.9	
	σ	7.1	6.5	3.2	4.5	7.4	6.0	3.7	
S. epidermidis	S_T	69.4	66.6	79.1	79.1	61.6	61.6	90.0	90.7
	Δ	8.0	7.6	5.2	5.2	8.1	6.9	3.1	4.1
	σ	9.1	9.2	6.4	6.4	8.3	9.1	4.0	6.2
S. saprophyticus CCM 883	S_T	68.7	64.0	80.7	66.8	54.5	59.8	68.2	92.4
	Δ	7.9	2.2	–	–	[illegible]	6.0	2.2	2.0
	σ	9.5	3.5	–	–	3.6	8.5	3.6	2.8

S_T mean transformed Yule correlation coefficient. $\bar{\Delta}$ mean deviation from mean, σ stand. of deviation

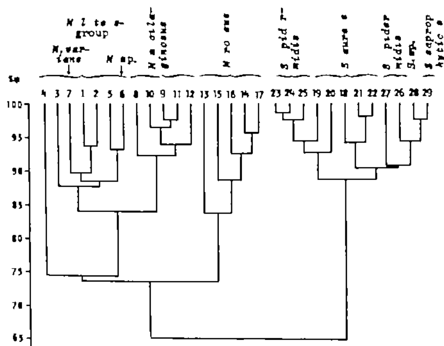


Fig 2 Phenogram of fatty acid contents of Micrococcus *and* Staphylococcus *strains.* The phenogram was obtained from the primary similarity matrix (Table 4) by unweighted pair group analysis according to *Sokal & Michener* (27)

formation of the data matrix by the formula $y = \ln(x + 1)$ entails that small peaks contribute relatively more to classification than their arithmetic values would ordinarily do. On the other hand, the logarithmic transformation reduces the classification value of quantitatively dominating peaks like 15:0-i, 15:0-a, 16:0-i, 16:0 17:0-a, and 18:0

Distinction of Genera

The classification of all strains included in this study was well-established in advance. The per cent(G+C) of DNA is either high corresponding to the range of the genus *Micrococcus* (56–75) or low corresponding to the range for the genus *Staphylococcus* (30–40) (17 Table 2) It appears from the present study that fatty acid composition renders a sharp distinction between the two genera, similar to the separation afforded by DNA base composition.

M. mucilaginosus *and* M roseus. The strains of these two species have been identified by conventional means, *M mucilaginosus* amongst others having a typical texture and *M roseus* being red pigmented. In addition modern biochemistry has substantiated the species allocation of these strains. *M mucilaginosus* has a characteristic rather low per cent(G+C) and L-Lys-L-Ala or L-Lys-L-Ser (L-Ala) peptidoglycans, whereas the peptidoglycan of *M roseus* is L-Lys-L-Ala$_3$. (Table 2) The peptidoglycan composition has been determined for all micrococci, except *M mucilaginosus* 4239/69 This strain belongs to the collection of strains examined during the re isolation of the species (2) and has 59 per cent(G+C) typical of the low range in *M mucilaginosus* Thus, the species identities of all these strains are firmly established. Accordingly by all procedures of interpretation, their fatty acid composition follows very clearly their preconceived differentiation into two well-defined entities.

M luteus *and the* "M. luteus-*group*" The species *M luteus* which until recently has been heterogeneous, was redefined by *Kocur*

et al. (18) and *Schleifer et al.* (23). All strains previously classified as *M. luteus* have here been included in the "*M. luteus*-group". These have 66–73 per cent(G+C), a yellow water-insoluble pigment, do not attack glucose, and have a peptidoglycan composition of the L-Lys-peptide subunit type. As partly seen from Table 2, all strains examined as *M. luteus* qualify. As detailed under *Results* the *M. luteus* strains form a heterogeneous cluster separate from *M. mucilaginosus* and *M. roseus*. Most of the phenogram heterogeneity of *M. luteus* (Fig. 2) is due to the abnormal affinity of strain no. 4 (CCM 1355) which can be explained by its particularly low similarity coefficient (Table 4) to strain CCM 210 which is the most recently proposed neotype strain of *M. luteus* (18). In terms of fatty acid composition, strain CCM 210 is not completely typical of the species (Table 3). It contains relatively little 15:0-i and exhibits a trace of 18:0-i (not encountered in any of the other *M. luteus* isolates). It nevertheless enters the *M. luteus* cluster in a central position (Fig. 2, strain 1). The strain CCM 810, presently listed as *M.* sp. according to *Schleifer & Kandler* (22), was previously proposed as neotype strain for *M. luteus* (4). It has later been revealed that CCM 810 differs from most other isolates of *M. luteus* (17, 23): its peptidoglycan type deviates (Table 2) and it does not provide any transformants in the *M. luteus* system (15, 23). Consequently CCM 810 has been considered unsuitable as a neotype strain. The single strain of *M. varians*, CCM 268, has many characteristics similar to *M. luteus* but it characteristically attacks glucose oxydatively, contains the L-Lys-L-Ala$_{3-4}$ type peptidoglycan (21, 23) and does not provide genetic transformation in the *M. luteus* system (5, 22, 23). Thus, both the *M.* sp. CCM 810 and *M. varians* CCM 268 differ significantly from *M. luteus* genetically. It appears also that the fatty acid composition (Table 3) of these two strains differs from that of *M. luteus*. Thus, *M.* sp. is the only *Micrococcus* strain with only trace amounts of the acids 14:0-i and 14:0. *M. varians* contains 20:0-i in significant amounts (3 per cent), in contrast to the trace quantities in *M. luteus*. Despite these differences, the two strains CCM 268 and CCM 810 enter the "*M. luteus*-group" cluster (Fig. 2).

Staphylococcal species. As appears from Table 2, the two species *S. aureus* and *S. epidermidis* are rather similar in characteristics otherwise considered important for the taxonomy within the *Micrococcaceae*. One of the *S. aureus* strains has the L-Lys-Gly$_{5-6}$ peptidoglycan pattern and the ribitol teichoic acid which are typical of this species. As regards the other strains of this species, these characteristics are unknown, but the identity was considered clear-cut due to the coagulase positivity. The strains of *S. epidermidis* and *S. saprophyticus* were coagulase negative and contained the characteristic peptidoglycan and teichoic acid types (Table 2). It would appear from the fatty acid contents (Table 3), similarity matrix (Table 4) and the phenogram (Fig. 2) that there is relatively little variation of fatty acid composition within the whole genus *Staphylococcus*. There is overlapping between preconceived species without clear distinction even of *S. aureus*. The separation of non-*S. aureus* strains into two clusters may be most important. The many biotypes in this group have led to markedly different suggestions concerning the classification of these organisms. The three coagulase negative staphylococci clustering together with *S. saprophyticus* in the phenogram (Fig. 2) have previously been named *S. lactis* (25): by traditional biochemical-cultural tests, they were different from the strains nos. 23, 24, and 25 which cluster together in another group of coagulase negative strains. The latter group of three strains has been identified by *Schleifer & Kocur* (22) as *S. epidermidis sensu stricto*, their subgroup II A, on the basis of biochemical-cultural characteristics, their peptidoglycan composition and their teichoic acid type. At the two latest meetings of the Subcommittee (29, 30) both *S. lactis* and *S. saprophyticus* were considered to be valid, recognizable entities.

Conclusion

It is important to note that fatty acid composition is not sufficient to distinguish between staphylococci. On the whole, however fatty acid content reflects species differentiation established by other procedures and thus is a valuable taxonomic criterion also in *Micrococcaceae*. Characteristic traits for the separation of the genera *Micrococcus* and *Staphylococcus* and the micrococcal species have been disclosed. The differentiation was supported by numerical analysis which turned out to be valuable in the evaluation of the GLC data.

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ELECTRON MICROSCOPY OF *BORRELIA MERIONESI* AND *BORRELIA RECURRENTIS*

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Borrelia merionesi and *Borrelia recurrentis* were studied in the electron microscope by means of negative staining and sectioning techniques. The cells of the two species had similar morphology. The cells were regularly waved and had pointed ends. Each organism had two bundles of flagella, one bundle originating from each end of the cell and each bundle consisting of 15–20 flagella. The flagella were twined together with the cytoplasmic body and overlapped in the middle region of the cell. The dimensions and the structures of flagella isolated from *Borrelia* were similar to the corresponding structures of flagella isolated from treponemes and gram-positive bacteria. A model for the sequence of morphological changes observed during the division process of *Borrelia* is proposed.

Previous studies on the ultrastructure of cells of the genus *Borrelia* have mainly been carried out with the use of shadow casting and ultrathin sectioning techniques for specimen preparation (for ref. see *Felsenfeld* (7) and *Gagy* (8)). These studies have established the helical shape of the *Borrelia* cells and also that the cells are surrounded by two triple-layered membranes with the flagella, the locomotory fibrils, situated in between. The cytoplasm contains ribosomes, mesosomes, vacuoles and nuclear regions (7, 8).

During our work on the ultrastructure of treponemes and leptospirae (5, 11, 12, 14, 16) we felt it desirable to include some species of the genus *Borrelia* which also belongs to the order *Spirochaetales*. Using the routine methods of the present-day electron microscopy laboratory for negative staining and ultrathin sectioning it was anticipated that some further knowledge on the ultrastructure of *Borrelia* cells would be gained.

The present paper reports the results of these studies on *B. merionesi* and *B. recurrentis* with particular reference to morphological details regarding position and structure of the flagella and their insertion organelles. In addition, some observations on the process of division of the cells of *Borrelia* are presented.

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MATERIAL AND METHODS

Borrelia merionesi was obtained from Professor *P. Gruner*, Institut Pasteur, Paris, France. *Borrelia recurrentis* was obtained from Dr. *J. Friend*, Department of Parasitology, Liverpool School of Tropical Medicine, England. Both strains were maintained in mice by intraperitoneal passage every 3–4 days.

Preparations for electron microscopy were made from blood. Mice were bled on day 3 or day 4

after inoculation and the blood was pooled. After coagulation of the blood the serum was pipetted off. Some preparations were made from blood to which 1 mg heparin per ml blood was added. The blood was stored at 4 C for about 1 hour before centrifugation at 1500 rev/min for 1–2 minutes, after which the plasma was pipetted off.

Preparations for Negative Staining

Depending on the number of *Borrelia* cells in the serum or the plasma obtained negatively stained preparations were made either directly or after further concentration of organisms by centrifugation at 15000 *g* for 20 minutes and resuspension in a few drops of the remaining serum or plasma.

The procedures for preparation of negatively stained unfixed organisms stained with 1 per cent ammonium molybdate as well as for treatment of cells with Teepol or with *Myxobacter* AL-1 protease 1 (AL 1 enzyme) prior to staining were identical to those described previously (11)

Preparations for Sectioning

Fourteen mice infected with *B. merionesi* and 8 mice infected with *B. recurrentis* were bled and 1 mg heparin per ml blood was added. The blood was pooled and stored at 4 C for 1½ hours before centrifugation at 1500 rev/min for 5 min to sediment the blood cells. The plasma was pipetted off and centrifuged in cone-shaped centrifuge tubes at 15000 *g* for 20 minutes. Most of the plasma was pipetted off and discarded, but about 0.3 ml was left in the tube. 2–3 ml 3 per cent glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 was added carefully to each tube and the plasma was left to solidify at room temperature (20–23 C) for 30 minutes (2). Clumps of *Borrelia* cells embedded in the jellied plasma were carefully dissected out and blocks with cells were trimmed to about 1 mm³ in size. These blocks were fixed overnight in 1 per cent OsO_4 in veronal acetate buffer pH 7.2 containing 0.01 M $CaCl_2$, to which was added 10 per cent YAP medium (yeast extract, sodium acetate peptone medium 0.3 0.05 and 0.3 per cent, respectively of the Difco products). After a brief wash in the veronal buffer mentioned above the blocks were treated for 1 hour at room temperature in 2 per cent uranyl acetate (19) in the same buffer prior to dehydration in alcohol and propylene oxide (18) and Vestopal-W embedding (20). Sections were obtained and counterstained with uranyl and lead salts according to the general routine of the laboratory and electron microscopy on sectioned and negatively stained material was also performed as described previously (11). For the present study approximately 450 recordings were studied

RESULTS

The length of the organisms of both species varied between 12 and 17 μm for individual cells. Organisms which showed indications of being close to division generally were at least 18–19 μm long. The cells were regularly waved (Fig. 1) with a wavelength of 1.7 μm for *B. merionesi* and 1.8 μm for *B. recurrentis*. The amplitude was 0.3 μm for both species.

The cytoplasmic body of the cells had sharply pointed ends (Figs. 1, 2, 3). The width of the cells increased gradually for about two wavelengths and the diameter measured in the middle of the cells was 0.4–0.5 μm. *B. merionesi* seemed to be slightly thinner than *B. recurrentis*. The surface of all organisms was covered by an amorphous layer of rather constant thickness, about 8 nm (Figs. 2, 3).

The flagella were inserted subterminally at both ends of the cells. The outermost insertion was about 0.3 μm from the sharply pointed end of the cytoplasmic body (Figs. 2, 3). The exact number of flagella inserted at each end was impossible to determine but was generally between 15 and 20. The flagella were inserted in a region extending from 1.0–1.5 μm along the length of the cells (Figs. 2, 3). Composite pictures of whole organisms at a high magnification showed two bundles of flagella on each organism, one bundle originating at each end. The bundles were intertwined with the cytoplasmic body of the cells and the flagella of each bundle overlapped in the middle region of the cells. The flagella seemed to be attached to the cytoplasmic body only at the ends of the cells, where the insertion organelles of the flagella were observed. Insertions for flagella present in the middle region were only observed in cells showing indication of division.

Organisms treated with Teepol retained their wavy appearance (Fig. 4) although the outer membrane was destroyed and the flagella were torn out of the cytoplasm (Figs. 4, 5). The flagella consisted of a shaft with a diameter of about 15 nm, a hook about

Figs. 1–12 all show material negatively stained with 1 per cent ammonium molybdate. The bar on each micrograph represents 100 nm unless otherwise stated.

Fig 1 An unfixed cell of *B. recurrentis* covered by an amorphous outer layer (arrow) The organism is regularly waved. Note the pointed ends. × 15 000.

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50 nm long and 15 nm wide with a honeycombed substructure and a collar approximately 17–18 nm long and 9 nm wide, which connected the hook to the basal knob (Figs. 4 5 6) The shape and the dimension of the basal knob were usually obscured by adhering membranous debris. A few micrographs gave the impression that the knob consists of two thin plates in close apposition to each other The diameter of the plates was 30–35 nm.

Only flagella and membranous debris were left on the supporting film of the grid when *Borrelia* cells were treated with AL-1 enzyme for 4 minutes (Figs. 7 8, 9) The flagella were still connected to membranes, and as many as 16 flagella attached to one and the same membrane fragment were occasionally observed (Fig 7) The insertion areas were often seen as electron dense round plates with a diameter of about 30 nm, surrounded

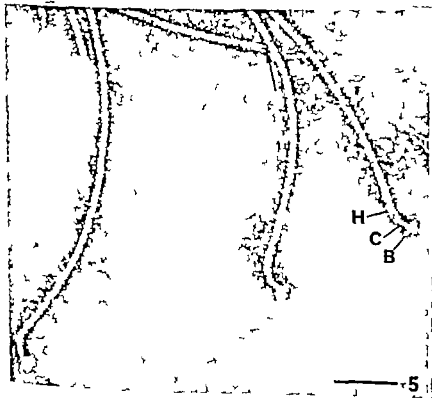
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Figs. 2–3 The cytoplasmic membranes (CM) the outer membranes (OM) and the amorphous surface layers (SL) of these cells are illustrated. Some of the insertion points (I) for the flagella (F) are also shown. Note that the width of the cells increases gradually from the tips along the cytoplasmic bodies. Unfixed organism of *B. merionesi* in Fig. 2 and *B recurrentis* in Fig. 3 × 90,000.

Fig 4 A cell of *B. merionesi* treated with Teepol for 2 minutes The flagella are torn out of the cytoplasm, but the cell has retained its wavy appearance. The flagella are seen to consist of a shaft (F) a hook (H) and a basal knob (B) × 90 000

Fig 5–6 Flagella isolated from cells of *B recurrentis* treated with Teepol for 4 minutes Basal knobs (B) collars (C) and hooks (H) are seen on the insertion parts of the flagella. Note that a small amount of negative staining material seems to have penetrated into the distal end of one of the flagella on Fig. 5 (arrow) × 160,000.

B H F B H F 4





by less electron dense rings about 10–15 nm wide (Figs. 8 9) A substructure could be seen in these rings on a few micrographs (Figs. 8, 9)

In micrographs of organisms treated with either Teepol or AL-1 enzyme, negative staining material seemed to have penetrated about 6 nm into the distal end of a few flagella (Fig 5) thus indicating that some flagella were hollow at their tips or possessed cores which were less resistant to the stain at this region.

The cells of the two *Borrelia* species studied appeared to divide by transverse fission. The earliest sign observed of preparation for division was a constriction of the cytoplasmic body in the middle of a long cell (Fig. 10) In this region the cytoplasmic body of such a cell was slightly thinner than the corresponding part of a non-dividing cell. At this stage flagella were found to be inserted at both ends of each of the two daughter cells (Fig 10) Flagella inserted at the ends of the "mother cell" were seen to stretch beyond the division site in the interspace between the cytoplasmic membrane and the outer membrane. Dividing cells in which the two new cells were completely separated by their cytoplasmic membranes, but still connected by a *mutual outer membrane* were also observed (Figs. 11 12) The new ends appeared truncated, but the tendency of the cytoplasmic bodies to increase in width along one or two wavelengths of the daughter cells was obvious (Fig 11) In organisms in which the distance between the two new cells was somewhat greater the cytoplasmic bodies of the daughter cells were found to possess tapered ends despite the fact that they were still covered by the same outer membrane (Fig 12)

Only sinusoidal pieces of *Borrelia* cells were included in each ultrathin section (Figs 13 14 15) The organisms were surrounded by a triple-layered membrane about 8 nm wide. Some adhering material was often observed on the outside of this membrane (Fig 14) Close to the inner leaflet of the outer membrane was an unstructured layer of medium electron density This layer was especially well seen in cross-sectioned organisms (Fig. 14) but was also observed in more longitudinally sectioned ones. The cytoplasm was surrounded by a triple-layered cytoplasmic membrane, about 7 nm wide (Figs. 14 15 16) An electron dense intermediate layer was observed in close apposition to the outer leaflet of the cytoplasmic membrane (Figs. 15 16) The flagella were situated between this intermediate layer and the unstructured layer adhering to the inner leaflet of the outer membrane. On several transversely sectioned organisms the flagella were seen to be arranged in two rows and they appeared to be uniformly dense (Figs. 15 16) The insertion part of the flagellum was extremely difficult to identify in sectioned cells. However occasionally ends of cells where the insertion parts of flagella may be conjectured were observed (Fig 13)

DISCUSSION

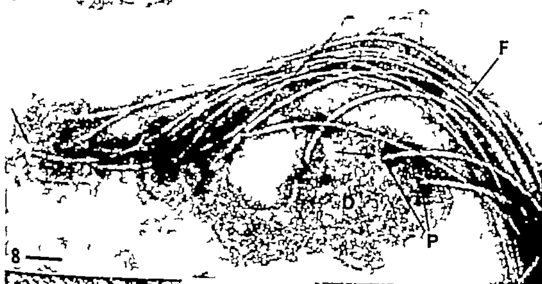
The results of the present investigation on the anatomy of cells of *B meriones*i and *B. recurrentis* show clearly that flagella released from cells of these organisms after detergent or enzyme treatment possess organelles the structures and dimensions of which are identical with those of flagella isolated from cells of various species of *Treponema* (4 10 11 12 13 14 15) *Spirochaeta* (9 17) and gram-positive bacteria (see *De Pamphilis &*

Figs. 7–9 all show material obtained from cells treated with *Myxobacter* AL-1 protease I for 4 minutes.

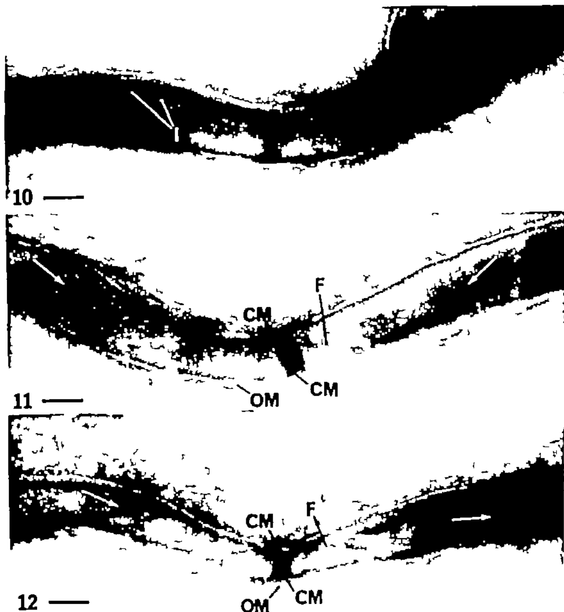
Fig 7 A tuft of 16 flagella attached to one membrane fragment from a preparation of *B. merionesi* cells. × 90 000

Fig 8–9 Membranous debris (D) and flagella (F) are the only remnants after treatment of cells with the enzyme. Insertion areas for the flagella are seen as electron dense round plates (P) surrounded by rings of lesser electron density A substructure is present in some of the rings (arrows) Fig 8 is from a preparation of *B. merionesi* cells (× 90,000) and Fig 9 from a preparation of *B. recurrentis* cells (× 160,000)


7


F
D
P
8


P
F
P
P
D
9

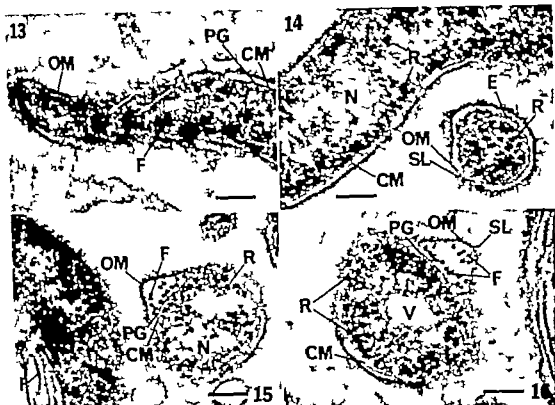


Figs. 10–12 all show dividing cells of *B. currentis.*

Fig. 10. A constriction of the cytoplasmic body in the middle of a cell. Insertion points of flagella of one daughter cell are seen at I. × 90,000

Figs. 11–12. Truncated end (Fig. 11) and tapered ends (Fig. 12) of daughter cells separated by their cytoplasmic membranes (CM) but covered by a mutual outer membrane (OM). Flagella (F) from the "mother cell" are seen in the interspace between the two membranes. Note insertion points for flagella of daughter cell on either side of the division site (arrows). × 90,000.

Adler (6) for further references). The flagella, however, appear to be thinner than flagella of the species mentioned due to the fact that the latter are generally coated with a sheath (3 nm thick), whereas all flagella isolated in the present study appeared to consist of naked flagellar shafts. In addition, the width of cross-cut flagella in sectioned cells



Figs. 13–16 Thin sections of *Borr lia* cells prepared as stated in the text. Adhering material (SL) is observed on the exterior of the outer membrane (OM) while on the interior part of this membrane a small amount of material of medium electron density (E) is visible. The flagella (F) are located in the interspace between the outer membrane (OM) and the intermediate layer (PG) which is found in close apposition to the outer leaf of the cytoplasmic membrane (CM) (see text). In some transversely sectioned cells the flagella (F) are seen to be arranged in two rows (Figs 15–16). Ribosomes (R) are distributed mainly at the periphery of the cells, while nuclear areas (N) are centrally located. A vacuole (V) is shown in the cytoplasm of the cell illustrated in Fig 16. One end of a longitudinally sectioned cell is presented in Fig 13. The arrows indicate what may possibly be insertion points of the flagella (F). Note the difference in width of the two cells shown in Fig 14. The transversely sectioned cell appears thinner and is probably sectioned close to the end of the organism. Figs. 13, 14 and 16 show *B. merionesi* cells and Fig. 15 cells of *B. caucasica* × 90,000.

of *Borrelia* also appears somewhat smaller than that of flagella of other organisms studied under similar conditions. It thus appears that flagella of *Borrelia* cells either are unsheated or possess sheaths of greater fragility than most other bacterial flagella.

Cells of the genus *Borrelia* obviously divide by binary fission.The formation of new flagella seems to occur early in the division process, as all dividing cells observed had four insertion regions for the flagella, one at each end of the organism and one on either side of the division site. As the flagella of the mother cell were seen to pass across the division site, it is regarded as most likely that the flagella inserted at each end of the division site are made *de novo*. After the daughter cells have been separated by their cytoplasmic membranes and presumably also by the apposed peptidoglycan layer (termed intermediate layer in the results) the next step in the division process is probably a tapering of the ends of the cytoplasmic bodies, without any observable increase of the distance between these ends and the outermost insertion points of the flagella. However the distance

between the tips of the cytoplasmic bodies of the daughter cells seems to increase simultaneously with the formation of the pointed ends. Finally when the new ends of the two daughter cells are pointed to the same extent as for mature cells, the cells presumably separate by fission of their outer membranes.

The extra contour observed on negatively stained organisms may be interpreted as an outer membrane covered by an exterior layer of presently unknown nature. The adhering material observed on the surface of sectioned cells may be remnants of such an outer layer.

The structures and dimensions of the cells of the investigated strains of *B. merionesi* and *B. recurrentis* are identical except that the wavelength of *B. merionesi* is slightly shorter than that of *B. recurrentis*.

According to *Geigy* (8) *B. recurrentis* and *B. duttoni* are morphologically indistinguishable and, in that author's opinion, *B. merionesi* is synonymous with *B. duttoni*. *Baltazard et al.* (3) on the other hand, found *B. merionesi* to be a species different from *B. duttoni*. However *Aeschlimann et al.* (1) studied ultrathin sections of cells of four different species of *Borrelia* obtained from blood of infected mice and found these to be identical. They concluded that probably all species of *Borrelia* found in the blood of mice were morphologically identical.

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[1] I am very thankful for the kind help and for the facilities placed at my disposal by civ.ing. *Aksel Birch Andersen* the Biophysics Department, and by d *H A ge Nielsen* the Treponematoses Department. My thanks are also due to *H J Skou gaard Jensen* D.V.M., who has been responsible for the maintenance of the strains of *Borr lia*. As usual I have had excellent assistance in electron microscopy by Mr *F Lau sen* and Mrs. *J Berg*. The expert photographic work has been performed by Miss *A G O ergaard* and Mr *F Lau sen*.

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ISOLATION AND BASIC CHARACTERIZATION OF TEMPERATURE-SENSITIVE MUTANTS FROM SEMLIKI FOREST VIRUS

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Sixteen temperature-sensitive mutants were isolated from N-methyl N' nitro-N-nitrosoguanidine treated Semliki Forest virus. Seven of the mutants were unable to synthesize virus specific RNA at the nonpermissive temperature (39° C) and were classified as RNA mutants as evidenced by temperature shift-up experiments they had defects only in early functions. Two mutants, designated RNA± made less virus specific RNA than the wild type virus. Six RNA mutants synthesized 42 S and 26 S RNA at 39° C in almost the same amounts and ratio as the wild type. Only one of them, ts-3 was unable to form the viral 140 S nucleocapsid at the nonpermissive temperature One of the RNA mutants, ts-1 made a higher proportion of 42 S RNA, presumably at the expense of 26 S RNA, both at 27 C and at 39 C. The synthesis of 42 S RNA and 26 S RNA in the Semliki Forest and Sindbis wild type virus infected cells was temperature dependent. There was an increased synthesis of 4 S RNA compared to 26 S RNA at 39 C, the situation being reversed t 27 C.

The main project of our group is to study the structure and replication of Semliki Forest virus, SFV (2 11 22 23 24 26 27 and 28) an arbo A virus, which belongs to the recently established togavirus group (1 31). *Because of the great value of the conditional* lethal mutants in these studies, the isolation of temperature-sensitive (ts) mutants from SFV was begun in a manner similar to that reported earlier for the closely related Sindbis virus (3)

The Sindbis virus mutants were first classi*fied into five non-overlapping complementa*tion groups (4) two of which were composed of mutants unable to produce viral RNA at the restrictive temperature. The other three complementation groups were linked to defects either in envelope nucleocapsid or a hypothetical maturation protein (4 5 6, 32) Later the RNA negative mutants were reclas*sified into three complementation groups* (14)

The biochemical properties of the *Sindbis* virus mutants have been studied intensively (5 6, 17 18 19 20 32) Temperature-sensitive mutants have also been isolated from Semliki Forest virus but their biochemical properties have been investigated less intensively (15 29)

In this paper we report the isolation and basic chara 16 obtained e

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The characterization of the mutants is biochemical rather than genetic because of the inherent difficulties in the recombination and complementation of these RNA viruses since the proteins are made by proteolytic cleavage of large precursor proteins (18, 19, 20). In an earlier report we showed that the cleavage of the precursor (NVP 68) of the envelope protein E_2 is inhibited in cells infected with ts-1 mutant (23). A more detailed report on the proteins synthesized by this and our other mutants at the nonpermissive temperature is in preparation.

MATERIALS AND METHODS

Virus and Cell Cultures

SFV prototype strain (10) was cloned three times before use both as the wild type virus and the source of the mutants. Other viruses used were a prototype strain of SFV received from the American Type Culture Collection and Sindbis virus, prototype EgAr 339 received from Dr. J. Casals (Yale Arborvirus Research Unit). Leukosis free secondary chick embryo fibroblasts (CE) received from this laboratory's leukosis free flock were grown on 60 or 50 mm petri dishes, 250 ml bottles (Falcon Plastics) or on plastic well plates (Linbro). Growth medium consisted of Eagles minimal essential medium (MEM) supplemented with 5 per cent inactivated calf serum and 10 per cent tryptose phosphate broth (Difco). 39 C was selected as the nonpermissive temperature and the incubator was kept in a 37 C hot room. The permissive temperature was 27 C. Both incubators were flushed constantly with 5 per cent CO_2.

Plaque Assay

For virus cloning and mutant isolation the plaque assay was performed using an agar overlay containing 0.9 per cent Bacto agar (Difco) in medium 199 supplemented with 5 per cent inactivated calf serum. When the plaque forming units (PFU) were determined 0.5 per cent carboxymethyl cellulose was used to solidify the overlay medium (30). The plaque counts were obtained after days at 39° C or after 4 days at 27 C.

One Cycle Growth Experiments

Cells were infected with 10 or 50 PFU/cell at the desired temperature. After 1 h adsorption the inoculum was removed and the cells were washed three times with prewarmed Hanks balanced salt solution. In some experiments the cells were re-washed 4 h post infection. The medium used was MEM containing 0.2 per cent bovine serum albumin (BSA, Armour) and actinomycin D 2 μg/ml as indicated. The released virus was harvested 8 (39° C) or 16 (27 C) h post infection. The cell debris was removed from the culture medium by low speed centrifugation and the supernatant was used for hemagglutination and infectivity assays.

Mutagen Treatment

N-methyl-N'-nitro-N-nitrosoguanidine treatment (100 μg/ml in 0.01 M tris buffer pH 7.6) of the wild type virus was performed as described by *Burge & Pfefferkorn* (3) except that the treatment period was 30 min at 20 C and that part of the sample was diluted immediately to 10^{-7} for plaque assay at 27 C and part was dialyzed over night against phosphate buffered saline (PBS) containing 0.5 per cent BSA, before the plaque assay. The infectivity recovered in the undialized and in the dialyzed sample was 6.7 per cent and 2.8 per cent respectively.

Isolation of Mutants

Well isolated plaques formed at 27 C under agar were collected and virus was eluted in 1 ml of PBS containing 0.5 per cent BSA. The ability of the eluted virus to grow at 39° C was tested using less than 1 PFU/cell in monolayers in plastic well plates. The cytopathic effect (CPE) was recorded at 24 h. The growth of those eluates with no CPE at 39 C was similarly tested at 27 C and the CPE was recorded at 48 h. Those eluates which showed CPE at 27 C only were assayed for infectivity at both temperatures. When the PFU titre at 27 C was 100 fold or more above that at 39 C, three well isolated plaques were collected from the 27 C assay plate. This was the second cloning of the mutants. To obtain the primary stock one plaque was grown in cell culture for 48 h at 27 C at an input multiplicity of infection of less than 1 PFU/cell. From these stocks the working stocks were grown for one further passage only at a multiplicity of infection 1 to 100 at 27 C.

Selection of Mutants

The primary stock was assayed at 39° C and at 27 C. When the PFU ratio at 39° C/27 C (plating efficiency) was 10 or lower the mutant was accepted and the leak yield determined. The cultures were infected with 10 PFU/cell at both temperatures. Culture fluids were collected at 8 h (39 C) and 16 h (27 C) and the infectivity of both harvests was determined by plaque assay at 27 C. Those mutants with a leak yield (virus yield at 39° C/virus yield at 27° C) of 10^{-3} or below were finally accepted for further studies.

Temperature Shift-up Experiments

Duplicate plates were infected at 27 C using 10 PFU/cell as described before. At 5 h post infection the culture medium was removed and replaced with fresh medium at 39 C and the incubation was continued at 39 C for another 5 h after which the culture fluid was harvested and the infectious irus formed was measured by plaque assay at 27 C. Another set of cultures used as controls was infected and maintained at 39 C for 8 h.

Hemagglutination Tit ation

The titration was performed at room tempera ture or at 39 C as indicated using 0.2 per cent goose erythrocytes at pH 5.8 (7)

RNA Synthesis

Confluent monolayers were infected at 50 PFU/cell in the presence of 2 μg/ml of actinomycin D as described above. At the times indicated ^{3}H uridine was added in 2 ml of fresh medium containing 20 μCi of ^{3}H-uridine (24–31 4 Ci/mmol, Amersham) and the incubation was continued for two more hours. After the pulse the cells were washed once with PBS and once with RSB-Na (0.01 M Tris pH 7 4 0.01 M NaCl, 0.0015 M $MgCl_2$) or with TSE (0 01 M Tris pH 7 4 0 15 M NaCl, 0.001 M EDTA Na) The monolayers were then disrupted in 2 per cent sodium dodecyl sul phate (SDS) in the final wash buffer and the cell lysate homogenized by passing through a small syringe needle. The acid insol ble radioactivity was measured from aliquots after precipitation with 5 per cent TCA (9) Sedimentation analysis was carried out on 15–30 per cent (w/w) sucrose gradients made in RSB-Na or TSE containing 0.1 per cent SDS in 18 ml capacity tubes (Spinco SW27 *rotor*) *Centrifugation was for 10 or 12 h* at 24 000 rev/min at 22 C. After centrifugation 0.5 ml fractions were collected from below and acid insoluble radioactivity was measured as described above.

Nucleoc psid Analysis

Cells infected with the mutants and kept at 39 C were exposed, from 5 to 5¼ h post infection to ^{14}C-amino acids, 20 μCi/dish (57 mCi/mAtom carbon) in MEM containing 1/10 of the normal amount of amino acids or 100 μCi/dish of ^{3}H-lysine (230 μCi/mmol) in MEM from which lysine was omitted. After the pulse the medium was removed and the cells were washed twice with MEM containing ten times the normal amino acids or lysine concentration. The cells were maintained in the chase medium for a further 30 min at 39° C. At the end of the chase the cells were washed with cold PBS, swollen in RSB-Na and disrupted in a Dounce homogenizer To the homogenate Triton X 100 was added to a final concentration of 1 per cent and the nuclei were pelleted at 250 g for 5 min. The cytoplasm was layered over 34 ml 15–30 per cent (w/w) sucrose gradients made in 0 05 M Tris pH 7 4 0.1 M NaCl 0.0015 M

TABLE 1 *Basic Characteristics of the 16 Temperatur -Sensitive M tants of SFV*

Virus	RNA phenotype	Reversion frequency	Leak yield	RNA synthesis at 39° C per cent of wild type	Nucleocapsid formation
ts-1	RNA	<3.6 × 10−[illegible]	1 7 × 10	102	+
ts-2	RNA	<3.5 × 10−[illegible]	9 1 × 10−[illegible]	66	+
ts-3	RNA	<1 1 × 10−	<5.9 × 10−[illegible]	82	—
ts-4	RNA	<1 4 × 10	2.1 × 10−[illegible]	0.9	ND
ts-5	RNA	<4.0 × 10	6.5 × 10	72	+
ts-6	RNA	<2.8 × 10−[illegible]	1.5 × 10[illegible]	1 1	ND
ts-7	RNA	<5.5 × 10−	1.5 × 10[illegible]	57	+
ts-8	RNA	<6 1 × 10^{-7}	1.5 × 10	1 2	ND
ts-9	RNA	<2.4 × 10	<3 7 × 10−[illegible]	0.9	ND
ts-10	RNA±	<2 2 × 10	<2.0 × 10−[illegible]	7 2	ND
ts-11	RNA	<6 7 × 10	8.0 × 10	4 4	ND
ts-12	RNA	<5 9 × 10	1.0 × 10	1.3	ND
ts-13	RNA±	<9 5 × 10	2.6 × 10−[illegible]	19	—
ts-14	RNA	2 4 × 10	1 4 × 10−	3.3	ND
ts-15	RNA	<1 0 10	<6.2 × 10−	44	+
ts-16	RNA	<6 9 × 10	5.9 × 10−[illegible]	78	+

ND = not done

$MgCl_2$ and centrifuged for 3 h at 4° C in a Spinco SW27 rotor at 25,000 rev/min. One ml fractions were collected from below and the acid insoluble radioactivity determined. In some cases double labelling was carried out using in addition to ^{14}C-amino acids also ^3H-uridine (100 µCi/dish). Except for washing, no attempt was made to stop the incorporation of uridine after the pulse.

RESULTS

Provisional Characterization of the Mutants

Altogether 340 plaques were collected from cloned, NTG treated Semliki Forest virus. A total of 16 ts-mutants with a reversion frequency lower than 10^{-4} and leak yield below 10^{-4} were obtained and coded ts-1 to ts-16 (Table 1). The mutants gave essentially the same yield as the wild type virus at 27° C which reached its maximum at about 16 h p.i. (Fig. 1). The working stocks showed the same reversion frequencies and leakiness as the primary stocks.

Hemagglutination titration of the wild type and all mutants after growth at 27° C were carried out both at room temperature and at 39° C. In every case including the wild type, the titer obtained at room temperature was 8 fold higher than that at 39° C. This indicates that none of the mutants have temperature-sensitive defects which affect the HA

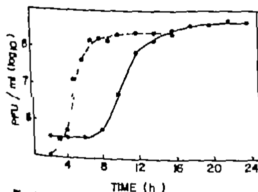


Fig. 1. One step growth curves of wild type SFV in secondary chick embryo fibroblast monolayers. At each point the culture fluid from duplicate cultures on 50 mm petri dishes (infected with 50 PFU/cell at zero time) was pooled and PFU titer was determined at 27° C. ○——○ growth at 39° C, ●——● growth at 27° C.

reaction. Nor did any of the mutants release hemagglutinins into the medium at 39° C, indicating that noninfectious, hemagglutinating particles were not produced.

The initial determination of the RNA phenotype was done at 39° C by giving a two hour pulse of ^3H uridine to the infected cells in the presence of actinomycin D. Under these conditions only the viral RNA is labelled (9). In the wild type infected cells the acid insoluble radioactivity was 50 to 100 times that of the uninfected control cells. Seven of the mutants (ts-1, ts-2, ts-3, ts-5, ts-7, ts-15, ts-16) incorporated ^3H uridine very efficiently, 40 to 100 per cent of the wild type value and were therefore classified RNA positive (RNA$^+$) mutants. The RNA negative (RNA$^-$) phenotype was assigned to those mutants (ts-4, ts-6, ts-8, ts-9, ts-12) giving only 1 to 2 per cent of the wild type incorporated radioactivity (Table 1). Four mutants (ts-10, ts-11, ts-13, ts-14) incorporated ^3H uridine clearly less than wild type virus but differed also from the background.

Temperature shift-up experiments were carried out with all the clearly RNA negative mutants and with those showing some ^3H uridine incorporation. The infected cells were first kept at 27° C for 5 h and thereafter shifted to 39° C for a further 5 h. Under these conditions those mutants which have defects only in functions needed early in the replication should grow normally. All the RNA$^-$ mutants replicated in the shifted cultures (Table 2). The ts-2, a RNA$^+$ mutant did not replicate after the shift up to 39° C. Two mutants ts-11 and ts-14 showing little RNA synthesis (Table 1) behaved exactly like the RNA$^-$ mutants and were therefore included in the RNA$^-$ group. Two mutants ts-10 and ts-13 capable of significantly higher RNA synthesis (7 and 19 per cent respectively) did not yield infectious virus in the shifted cultures. These mutants were classified into an intermediate group designated as RNA± (Table 1).

The temperature shift-down experiment in which the infected cultures were incubated for 4 h at 39° C and thereafter shifted to

TABLE 2. *Temperature Shift-up Experiment*

Virus	RNA phenotype	Plaque forming unit per cell	
		8 h at 39 C	5 h at ?7 C + 5 h at 39 C
ts-4	RNA	0.005	16
ts-6	RNA	0.005	83
ts-8	RNA	0 07	110
ts-9	RNA	0.003	3.8
ts-11	RNA	0.04	190
ts-12	RNA	0 4	150
ts-14	RNA	0.7	240
ts-10	RNA±	0 001	0.003
ts-13	RNA±	0.001	0 002
ts-2	RNA	0.008	0 004
wild type	RNA	180	140

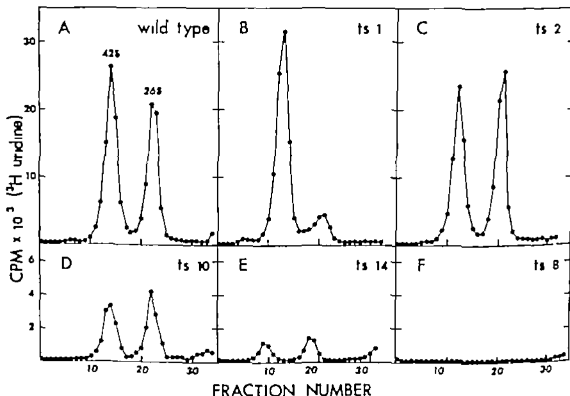


Fig 2 Sucrose gradient nalys of w ld type (A) ts-1 (B) ts-2 (C) ts-10 (D) ts-14 (E) and ts-8 (F) mutants of Seml ki Forest iru grown at 3 C Two petri dishes of secondary CE cells infected with 50 PFU/cell and maintained *in MEM + 0 2 per cent BSA + 2 μg/ml of actinomycin* D were pulsed from 3 to 5 h p w th 10 μCi ml of H-uridine At the end of the pulse ell were washed, taken into RSB-Na containing per ent SDS d srupted and analyzed in 15 30 per cent sucrose made in RSB-Na containing 0 1 per ent SDS a described in Materials and Methods. Centrifugation was for 10 h at 24,000 rev/min in SW 27 rotor 18 ml tubes) at 22 C. S-values were determined using ribosomal 28 S and 18 S RNA absorbanc m rkers Bottom at left

TABLE 3 *Synthesis of 42 S and 26 S RNA in Semliki Forest Virus ts-mutant Infected Cells at 39° C*

Virus	RNA phenotype	^{3}H-uridine incorporated into 10 cells			42S/26S RNA ratio
		Total CPM × 10^{-3}	Per cent in 42 S RNA	Per cent in 26 S RNA	
ts-1	RNA	103	69 7	9.0	7 7
ts-2	RNA	77.3	41.0	35.6	1.2
ts-3	RNA	85.6	41 1	34.9	1.2
ts-5	RNA	64 4	31.3	5 6	2.0
ts-7	RNA	65.6	46.2	28.8	1.6
ts-10	RNA±	7.3	36.3	32 7	1 1
ts-11	RNA	4.5	40.0	22.3	1.8
ts-13	RNA±	17.8	49.8	24 9	2.0
ts-14	RNA	3 0	31.6	28.9	1 1
ts-15	RNA	4[illegible]2	42.6	33.8	1.3
ts-16	RNA	71.8	48.3	27.8	1 7
wild type	RNA	90 0	47.0	31.8	1.5

27 C for a further 4 h was carried out with ts-10 and ts-13 The yields of the shifted cultures were 1 4 and 0 002 PFU/cell for ts-10 and ts-13 respectively According to these results both these mutants seem to have defects in both the early and late functions of replication.

Synthesis of the Virus Specific RNAs at 39° C

The RNA species synthesized by the mutants in actinomycin D treated cells at the nonpermissive temperature were determined using a two hour pulse of H-uridine beginning 3 h post infection. The sucrose gradient profiles of the RNAs of wild type virus and some of the mutants are shown in Fig 2 Under these conditions, in arbo A virus infected cells, there are two major virus specific single-stranded RNAs formed, which sediment at 42 S and 26 S (8) The former is the viral genome and the latter represents gene duplication of 1/3 of the 42 S RNA (21) The replicative intermediate sedimenting between 20 and 29 S (9) and the single stranded 20 S, 33 S and 38 S RNAs also found in SFV infected cells account for only a small amount of the total RNA (13 16) Repeated experiments with the wild type virus have shown that at most 10 per cent of the RNA sedimenting at 26 S is resistant to pancreatic RNase (5 µg/ml 30 min at 37 C) due to a contamination by replicative intermediates. Most of this RNA represents, however genuine single stranded 26 S RNA and is referred to as such

The acid insoluble radioactivity sedimenting at 42 S and 26 S was calculated from the sucrose gradient fractions and compared to the total acid insoluble radioactivity recovered from the gradient. The results for all RNA RNA± and also for two RNA mutants showing slight RNA synthesis (Table 1) are expressed per 10^6 cells and given in Table 3

For the wild type a value of 1.5 for the 42 S/26 S RNA ratio was obtained. In similar two hour pulses with ^{3}H-uridine the value varied from 1.5 to 2.5 (4 experiments) All but one of the mutants tested had 42 S/26 S RNA ratios between 1 and 2. The only exception was ts-1 which synthesized considerably less RNA sedimenting at 26 S although the total amount of RNA synthesized was not reduced. About 1½ times more 42 S RNA was made than in wild type infected cells, suggesting that the reduced synthesis of 26 S RNA was compensated for by an increased synthesis of 42 S RNA and resulting in a 42 S/26 S RNA ratio of 7 7 in this particular experiment. The overall variations in the

RNA ratios was 6.1 to 10.7 (4 experiments). About 20 per cent of the radioactivity at 26 S was resistant to pancreatic RNase (5 μg/ml 30 min at 37 C) suggesting that the actual amount of 26 S RNA was less than that indicated in Table 3.

Temperature Dependence of the 42 S/26 S RNA Ratio

Because the ts-1 mutant was clearly deficient in the synthesis of the 26 S RNA at the nonpermissive temperature, RNA synthesis at both 27 C and 39 C was studied more closely. In these experiments two other mutants, ts-2 and ts-3, as well as two different strains of SFV and one strain of Sindbis were included. A two hour pulse of ^{3}H-uridine was given at 3 h and 6 h post infection at 39 C and 27 C respectively and the total virus specific cellular RNAs were analyzed by sucrose gradient centrifugation in TSE (Table 4). 42 S and 26 S RNA accounted for 70–90 per cent of the total acid insoluble radioactivity at both temperatures. A striking difference in the 42 S/26 S RNA ratio at 27 C and 39 C was observed with all the viruses tested. At the lower temperature more 26 S RNA and less 42 S RNA was synthesized than at the higher temperature. The increased amount in 26 S RNA at 27 C could not be accounted for by contamination with the replicative intermediate since the level of RNase resistant material remained at 10 per cent of the total again.

The 42 S/26 S RNA ratio for the same stock virus varied to some extent, but the difference obtained between the two temperatures was always 3 fold or greater. The ts-2 and ts-3 mutants showed wild type RNA synthesis patterns at 39 C and 27 C. The ts-1 mutant produced more 42 S RNA at both temperatures than any of the other viruses studied. It still exhibited the same difference in ratios between the two temperatures as the other mutants and the wild type virus. Several temperature shift-up and -down experiments were carried out with ts-1 and the wild type. All of them confirmed the temperature dependence of the 42 S/26 S RNA ratio for both mutant and wild type. Inhibition of protein synthesis by cycloheximide (100 μg/ml) before the cultures were shifted down gave the same 42 S/26 S RNA ratio as in the absence of cycloheximide. This shows that the alteration in RNA synthesis does not require protein synthesis.

Nucleocapsid Formation

In SFV infected cells the viral nucleocapsid which consists of the 42 S RNA genome and about 240 identical capsid proteins, MW 33,000 (10, 12), is formed from a polysomal precursor (27) within 15 min (25). The ability of the mutants to form viral nu-

TABLE 4 *Effect of the Temperature on the Synthesis of Semliki Forest Virus 42 S and 26 S RNAs in CE Cells*

Virus strain	Temperature during virus growth and period of labelling with ^{3}H-uridine					
	39 C pulse 3 – 5 hours p.i.			27 C pulse 6 – 8 hours p.i.		
	Per cent in 42 S RNA	Per cent in 26 S RNA	42 S/26 S	Per cent in 42 S RNA	Per cent in 26 S RNA	42 S/26 S
Wild type (cloned)	57.0	24.3	2.3	31.9	42.5	0.75
ts-1	73.8	6.9	10.7	57.5	21.9	2.62
ts-2	55.[illegible]	24.6	2.2	27.7	41.2	0.67
ts-3	55.2	25.5	2.2	26.9	49.3	0.55
SFV prototype strain 1	65.9	18.9	3.5	22.3	59.8	0.37
SFV prototype strain 2	54.1	23.9	2.3	24.1	55.4	0.44
Sindbis	38.9	37.3	1.0	18.6	55.0	0.34

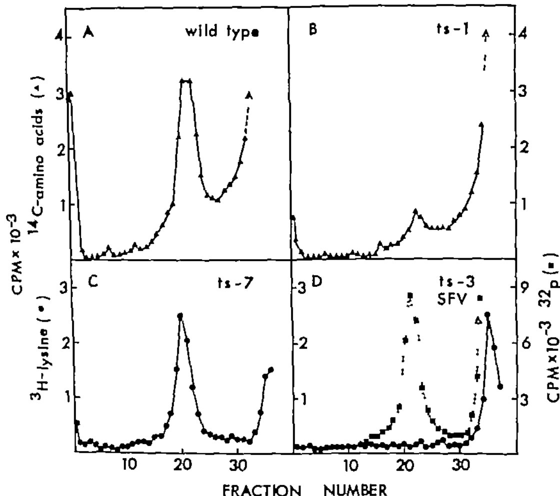


Fig. 3 Nucleocapsid synthesis in CE cells infected with wild type (A) ts-1 (B) ts-7 (C) and ts-3 (D) mutants of Semliki Forest virus. Infected cells were pulsed with ^{14}C-amino acids (10 μCi/ml) or with ^{3}H-lysine (50 μCi/ml) for 30 min at 5 h p.i. followed by 30 min chase. The preparation of the cytoplasmic extract in 1 per cent Triton X-100 was as described in Materials and Methods. Centrifugation was for 3 h at 25,000 rev/min in SW27 rotor (38 ml tubes) at 4 C. SFV labelled with ^{32}P was treated with 1 per cent Triton X 100 to release the nucleocapsid and centrifuged in a separate tube. ^{14}C: ▲, ^{3}H: ● ^{32}P ■ Sedimentation was from right to left.

cleocapsid at the nonpermissive temperature was tested by giving a 30 min pulse of ^{14}C-amino acids of ^{3}H-lysine to the infected cells at 5 h post infection in the presence of actinomycin D. After the pulse, nonradioactive amino acids were added and the incubation was continued for 30 min at 39 C. The cytoplasm was collected and analyzed in 15 to 30 per cent sucrose gradient after solubilization of the membranes by 1 per cent Triton X 100. Under these conditions radioactive material sedimenting with a peak at about 140 S consists almost exclusively of nucleocapsids (27) In Fig 3 sucrose gradient profiles of the cytoplasmic nucleocapsid of wild type virus, ts-1 ts-3 and ts-7 are presented. In the wild type infected cells 54 per cent of the sedimenting (>20 S) ^{14}C-amino acid label was found between 120 S and 160 S. The corresponding figure for ts-7 one of the nucleocapsid positive mutants, was 54 per cent with ^{14}C-amino acids and 63 per cent with ^{3}H-lysine label. Ts-3 was shown to be almost as efficient a producer of nucleo-

capsid (54 per cent ^{3}H lysine label sedimented between 120–160 S). Mutants ts-1 (^{14}C 43 per cent) ts-2 (^{3}H 36 per cent) ts-15 (^{14}C 31 per cent) and ts-16 (^{14}C 45 per cent) were somewhat less efficient in the capacity to form nucleocapsid. These were, however, classified as nucleocapsid positive mutants. Ts-3 clearly a RNA positive mutant, did not show any nucleocapsid peak (Fig. 3) nor did ts-13 which is a RNA± mutant. Double labelling with ^{3}H-uridine and ^{14}C-amino acids was performed with mutants ts-1, ts-2, ts-13, ts-15 and ts-16. The RNA from the 140 S peak was analyzed. In all cases it was largely (>70 per cent) 42 S RNA.

DISCUSSION

In the isolation of temperature-sensitive mutants from Semliki Forest virus reported in this paper, fairly stringent criteria were used for the back mutation frequency (10^{-4}) and for the leakiness (10^{-4}) in order to select stable mutants suitable for biochemical studies. This may have led us to select some double mutants. Their proportion should, however, be low since the same conditions and criteria were used as for the Sindbis virus mutants of Burge and Pfefferkorn most of which were single step mutants as shown by their ability to form complementation groups. Two of our mutants, ts-10 and ts-13 showed defects both in early and late functions in replication (Table 2) and are probably double mutants.

The main characteristics of the mutants are given in Table 1. Five of them are unable to synthesize detectable amounts of viral RNA at the nonpermissive temperature and showed only early function defects in temperature shift-up experiment. Two mutants, ts-11 and ts-14, which showed some RNA synthesis at the nonpermissive temperature were included in the RNA$^-$ group since they behaved in the shift up experiment like RNA negative mutants. Two other mutants, ts-10 and ts-13, synthesized somewhat more RNA. Neither of them replicated after a shift to the nonpermissive temperature thus showing a late function defect in addition to the obvious impairement of RNA synthesis. Since a considerable amount of RNA (7 and 19 per cent for ts-10 and ts-13 respectively) is formed at 39 C one would except at least some infectious virus to be made in cultures shifted down to the permissive temperature. This turned out to be so for ts-10. Interestingly enough, ts-13 did not show increased virus production in the shifted cultures despite its higher RNA synthesis. It is possible that the RNA formed at high temperature is either unable to direct viral protein synthesis or cannot form virus particles even at the lower temperature.

All the RNA$^+$ and RNA± mutants except ts-1 synthesized both 42 S and 26 S RNA at the nonpermissive temperature in the same ratio as the wild type virus. Ts-1 showed a clear increase in 42 S and decrease in 26 S RNA synthesis. When the temperature dependence of this mutation was studied in more detail an interesting property of the wild type was noticed. The 42 S/26 S RNA ratio was found to be temperature dependent: 2–3 times more 42 S RNA was produced at 39 C than at 27 C. This increased 42 S RNA synthesis was seemingly compensated by reduced synthesis of 26 S RNA. This observation was confirmed with two different strains of SFV and also with Sindbis virus. The temperature dependent change of the ts-1 42 S/26 S RNA ratio was of the same order of magnitude as that of the wild type. Anyhow it is obvious that the increased capacity of ts-1 to produce 42 S RNA at both temperatures is due to a mutation. The mutation causing more 42 S to be synthesized by ts-1 appears to be temperature independent and was detected only because ts-1 has another temperature sensitive mutation namely the inability to cleave the envelope protein precursor NVP 68 into E_2 (23). In Sindbis virus infected cells the 42 S/26 S RNA ratio can be altered by inhibiting the protein synthesis 1½ h post infection as shown by *Scheele* & *Pfefferkorn* (17). As a result much less 26 S RNA is produced. The authors suggest that a specific protein is

needed for the synthesis of 26 S RNA. We think that this "26 S protein" is altered in our ts-1 due to a mutation. In the Sindbis ts-24 a similar mutation has been suggested (17)

The temperature dependence of the 42 S/26 S RNA ratio and the properties of ts-1 could be explained by assuming that the normal "26 S protein" binds reversibly to the replicase or possibly to the template. This would allow transcription of either 26 S RNA or the whole 42 S RNA. The binding is more stable at the lower temperature resulting in a pronounced 26 S RNA synthesis while the reverse is true at the higher temperature. A mutation affecting the "26 S protein" could result in reduced affinity for the acceptor as would be the case with our mutant ts-1 and Sindbis mutant ts-24

The temperature dependence of the association constant of this protein may well be connected with the ability of arbo A viruses to grow at low (arthropods) as well as at high (mammalians and birds) temperatures.

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PROTEIN A PRODUCTION IN DIFFERENT STRAINS OF *STAPHYLOCOCCUS AUREUS* UNDER VARIED GROWTH CONDITIONS

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The protein A content of extracts of staphylococcal cells was estimated by the single radial immunodiffusion technique (Mancini technique) and by a haemagglutination test. The grouping of *S. aureus* strains according to protein A production using these methods was essentially similar to that based on their different affinity to FITC-labelled immunoglobulins. High yields of protein A were obtained from staphylococcal cells which were harvested after growth on solid medium and extracted in 0.07 M phosphate buffer at pH 5.9 for 10 days at room temperature. The presence of sodium chloride (7.5 per cent) in the medium suppressed the formation of protein A. The immunogenic properties of both protein A producing strains and protein A were dependent on the presence or absence of serum (IgG) in the medium. Due to the complex formation between protein A and IgG even highly purified protein A induced antibodies which reacted specifically with immunoglobulins when serum or blood had been present in the medium.

In previous studies (9, 10) the grouping of staphylococcal strains according to protein A production was based on their stainability in the fluorescent antibody test (FAT). In the case of staphylococci, the binding of labelled globulins is dominated by the strong affinity of protein A for the Fc part of IgG molecules (2, 6, 11) and therefore the prevalence of protein A producing strains could be studied by this technique.

The evaluation of the protein A production of a particular strain by the FAT was based on examination of a comparatively small number of cells, namely the number present in a thin smear suitable for fluorescence microscopy. In the present study the results obtained by the quantitative determination of protein A in the extracts of 60 representative strains were compared with those obtained in the previous studies.

The protein A production by 10 strains of *S. aureus* under varied growth conditions was also studied, and the different protein A preparations were characterized immunochemically.

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MATERIAL AND METHODS

Bacterial strains. Fifty-six strains of *Staphylococcus aureus* (*S. aureus*) were examined, which had presented different degrees of protein A reactivity in the FAT (10). Four strains used previously for studies of protein A reactivity (11, 12, 13) were included, *viz.* the protein A negative (pA neg) strain *S. aureus* Wood 46 (NCTC 10344) and three protein A positive (pA pos) strains: *S. aureus* Cowan I (NCTC 8530), 4972 and 8341.

Media. The fluid media used were trypsin broth (17) trypsin broth with 7.5 per cent NaCl Hartley's broth (3) and 4 per cent NZ amine type NAK medium (Sheffield Chemical) supplemented with 0.0005 per cent thiamine and 0 001 per cent niacin (4)

The solid media used were NZ amine medium (4 per cent NZ amine type NAK medium enriched with 0 0005 per cent thiamine and 0.001 per cent niacin) solidified with 1 per cent Noble Special Agar Bacto Phenol Red Mannitol Agar (NaCl concentration increased to 7.5 per cent) and broth peptone agar with one of the following four admixtures 0.1 per cent glucose 0.2 per cent $CaCl_2$ (medium used for phage typing of staphylococci) 0.2 per cent $CaCl_2$ + 7.5 per cent NaCl, or 10 per cent horse blood.

Crude protei A The bacteria were cultivated for 18 hours at 36 C, the broth cultures being shaken in an Orbital incubator IH 400 (Gallenkamp) After growth on solid medium the cells were harvested in 0.07 M phosphate buffer at pH 5.9 (0.5 ml per petri dish (9 cm diameter)) and the suspension was left at room temperature for 10 days. The suspension was then centrifuged and the supernatant used as crude protein A For special purposes, the solution was sterilized by passage through a Millipore® filter After growth in broth (100 ml) the cells were harvested by centrifugation and the pellet washed three times in phosphate buffer pH 5.9 and resuspended in 3 ml of the same buffer The suspensions were left for 10 days at room temperature. For all suspensions the iable counts were determined on the first day

Purified protei A Preparations of crude protein A from selected strains (4972 and Cowan 1) were purified as described previously (13) The protein concentration of purified protein A solutions was determined according to the method of *Lowry et al* (15) *Human serum albumin was* used as reference.

Quantitative protein A determination a) *Modified Mancini technique* Single radial immunodiffusion according to *M ncini et al.* (16) was modified for protein A determinations. The gel was 1 per cent agarose (L Industrie Biologique Française in barbiturate buffer at pH 8.6, ionic strength 0 1 to which 5 μl normal porcine serum per ml w added (corresponding to 0.6 μl porcine serum per cm^2 gel area) Four dilutions of each protein A preparation were applied in amounts of 5 μl, and the plates were incubated in a moist chamber at room temperature The diameter of the circular precipitate was measured by means of a dial caliper (Kafer 0 01 mm) b) *Protei A haemagglut nation t t* The test was performed according to *Sjöquist & Stålenheim* (19) as modified by *Kronvall* (7)

Double diffusion g gel (Ouchterlony analysis) (11) *immunoelectrophoretic analysis* (12) *analytical ultracentrif gation* (13) *immunization proc d res* (13) and *absorption experiments* (12) were performed as described previously in the papers indicated.

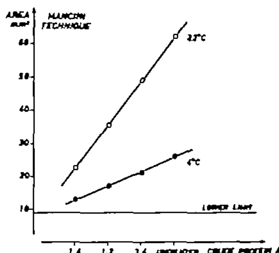


Fig 1 Quantitative protein A determination by modified Mancini technique Four dilutions of each protein A preparation were examined. The figure illustrates the linear correlation between the area of the precipitate and the concentration of protein A. The two extracts examined were obtained from identical suspensions of *S aureus* Cowan I after extraction for 10 days at room temperature (22 C) and 4 C, respectively

RESULTS

Methods for quantitative protein A determination. a) *Modified Mancini technique* The principles of single radial immunodiffusion established by *Mancini et al* (16) for quantitative determination of antigen concentration were found to apply also for quantitative protein A determination. This means that a linear relation was demonstrated between the area of the protein A/IgG precipitate and the protein A concentration when the precipitate had reached its final size (Fig 1) Only the larger precipitates required more than 24 hours to reach the constant state. A standard curve was determined for purified protein A of Cowan 1 at concentrations between 100 and 500 μg/ml, which in the test system chosen gave areas of precipitates from 15 to 50 mm^2 Each point was fixed as the mean

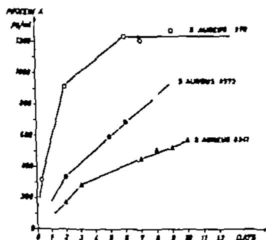


Fig 2 Quantitative protein A determination by modified Mancini technique. The amount of protein A released from the bacteria at room temperature (22° C) increases with time. The figure illustrates the amount obtained from *S aureus* 4972 in two different experiments and from *S aureus* 4341 in one experiment. In general, the amount of protein A recovered increased with time for at least 8–10 days.

of 10 determinations. The test sample was examined at four dilutions, the diameter of each precipitate was measured in duplicate at right angles, and the corresponding area was calculated from the mean. The protein A content was determined by reference to the standard curve (standard error 16 per cent). Slightly more precise information (standard error 14 per cent) could be achieved by determination of the protein A content from the ratio between the slope of the regression line of the test sample and that of the standard preparation (provided that the larger precipitates (> 80 mm) were omitted from the calculations and that test sample and standard preparation were examined on the same plate). b) *Haemagglutination test* The modification of the sensitized sheep red cell test for protein A determination (19) described by *Kronvall* (7) gave reproducible results and detected protein A even at a concentration of 20–30 ng protein A per ml. Throughout this study an anti protein A antiserum was used which had been prepared against whole cells of *S aureus* Cowan 1

TABLE 1 *Yield of Protein A per 10 Cells After Boiling for ½ Hour or Extraction for 10 Days at Room Temperature*

S aureus strain	µg protein A per 10^{10} cells 22 C 10 days	100 C 30 min
Cowan 1	204	49
4972	206	44
4291	175	81
1444	84	32
2024	78	24
Wood 46	0	0

(NZ amine medium) i.e. the same kind of serum as used by *Kronvall*.

Preparation of crude protein A *Jensen* prepared "antigen A" by extraction of staphylococcal cells at room temperature for 14 days (5 p. 24) or by boiling the bacteria for ½ hour (5 p. 98). In preliminary experiments it was examined whether a change of these procedures would influence the yield of protein A gained from a given amount of bacterial cells. By extraction at 4 C instead of room temperature the yield of protein A was reduced considerably (Fig 1). The amount of protein A which was released from the cells increased with time (Fig 2) and therefore in comparative experiments it was essential to keep the extraction time constant. Boiling of the bacteria gave a low yield (Table 1) and changed the protein A reactivity qualitatively since the precipitates obtained with boiled extracts were indistinct and weak. The standard procedure for preparation of crude protein A in the present experiments (see Material and Methods) was chosen on the basis of these experiences.

Comparison of protein A production in 60 strains of S. aureus. A crude extract was prepared from each strain and the protein A content was measured by single radial immunodiffusion and by the haemagglutination test. There was positive correlation between the results obtained with the two techniques (Fig. 3).

The amount of protein A formed per 10^{10} cells was calculated for each strain employing

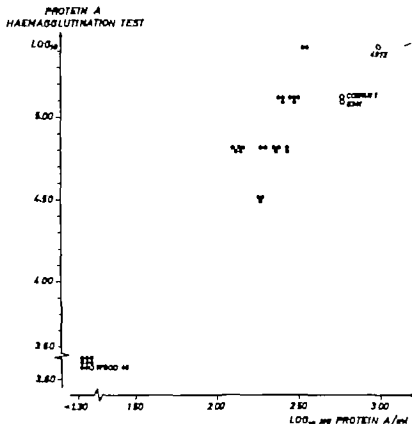


Fig 3 Protein A production in 55 strains of *S aureus* by growth on nutrient agar containing 10 per cent horse blood. Crude extracts were tested both in the haemagglutination test and by the modified Mancini technique. For each extract the log titre of the haemagglutination test is plotted on the ordinate and μg protein A per ml ($\log_{10}$) determined by the Mancini technique on the abscissa. There is positive correlation between the results obtained with the two techniques. Open circles indicate the strains used in previous studies (11 12, 13)

the results obtained by the Mancini technique. These values were plotted against the five groups of protein A reactivity found by the fluorescent antibody test (FAT) (Fig 4) The results are in accordance. *S aureus* Cowan 1 which is the strain commonly used for protein A production, belongs to the group I *i.e* + + + + reactivity in FAT In the present selected material about 10 strains were found to produce a similar amount or even more.

Yield of protein A after growth on different media. The amount of protein A formed by a particular strain when grown on different media was examined for 10 strains. Abundant growth and a high yield were obtained on solid NZ amine NAK medium enriched with B vitamins. The superiority of this medium was most pronounced for strains which produce large amounts of protein A. For six strains tested, the lowest yield was obtained from cells which had been grown on medium containing 7.5 per cent NaCl. The yield of protein A in the extracts of *S aureus* Cowan 1 and 4972 is shown in Table 2. Extracts of cells which had been grown on NZ amine medium or on nutrient agar + 10 per cent horse blood were prepared several times, and the yield varied noticeably (*e.g* day-to-day variation for protein A production of Cowan 1 grown on horse blood agar was about ± 30 per cent of the mean 109 μg/10 cells) Cells grown in broth gave a low yield especially when they had been washed before the ex

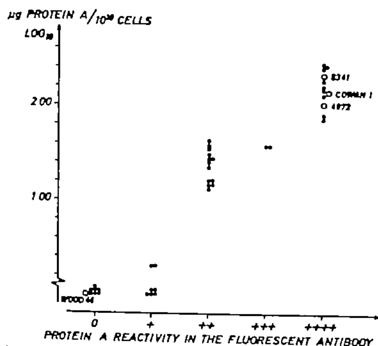


Fig. 4 Protein A production in 60 strains of *S. aureus* by growth on nutrient agar containing 10 per cent horse blood. The amount of protein A formed per 10¹⁰ cells was calculated for each strain and plotted on the ordinate. The five groups found by the FAT are indicated on the abscissa. FAT group I corresponds to + + + + reactivity in FAT, the intermediate groups corresponds to +, + + and + + + and group V contains the pA neg strains. Open circles indicate the strains used in previous studies (11, 12, 13)

extraction procedure was started (Cowan 1: 45–46 μg protein A per 10¹⁰ cells; 4972: 15–37 μg protein A per 10¹⁰ cells).

Cells with a diminished release of protein A into the extract showed a decreased binding of IgG as judged by absorption experiments including subsequent immunoelectrophoretic analysis of the absorbed sera.

Immunochemical characterization of protein A from different staphylococcal strains. Experiments were designed to compare protein A preparations obtained from different strains and to compare protein A antigens produced by a particular strain under varied growth conditions. Generally the protein A reactivity of crude extracts differed only quantitatively. An indication of qualitative differences was occasionally observed, either as a discrepancy between the reaction in the Mancini technique and the haemagglutination test, or as a less distinct precipitation reaction with normal porcine serum.

Protein A preparations from *S. aureus* Cowan 1 and 4972 were studied in more detail. Purified protein A preparations (13) obtained from cells both after growth on NZ amine medium and on nutrient agar containing 10 per cent horse blood were found to give similar sedimentation coefficients by analytical ultracentrifugation. Analogous precipitation patterns by double diffusion in agar gel were formed with antisera to whole cells and crude extracts of staphylococci and with normal sera from rabbit, swine and man. There was no indication of differing immunogenic properties of these purified protein A preparations when antisera raised in rabbits were tested against homologous and heterologous antigens. However, in a control experiment, a weak precipitation line was seen between the crude extract of the pA neg strain Wood 46 and antiserum to purified protein A (Cowan 1). Both Wood 46 and Cowan 1 had been grown on nutrient

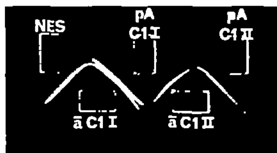


Fig 5 Double diffusion in agar gel (Ouchterlony analysis) NES normal equine serum diluted 1 100 pA C1 I purified protein A from *S aureus* Cowan 1 after growth on nutrient agar containing 10 per cent horse blood pA C1 II: purified protein A from *S aureus* Cowan 1 after growth on NZ amine medium ā C1 I rabbit antiserum against pA C1 I ā C1 II rabbit antiserum against pA C1 II ā C1 I contained some antibodies which precipitated a component in NES (IgG) and other antibodies which formed a line with pA C1 I the latter line gave reaction of identity with the line formed between pA C1 I and ā C1 II

agar containing horse blood. With a proper arrangement of the reactants in Ouchterlony analysis, the anti-protein A antiserum could be shown to produce two lines with the homologous antigen one giving reaction of identity with a line produced against horse serum, the other giving reaction of identity with the line produced with normal porcine serum or the line produced between protein A from cells grown on NZ amine medium and its homologous antiserum (Fig 5) Immunoelectrophoretic analysis using normal equine serum as antigen demonstrated that the anti protein A antiserum reacted specifically with equine IgG

Antibodies to equine IgG in rabbit anti staphylococcal sera. Investigation of all rabbit antistaphylococcal sera used in previous studies showed that all protein A producing staphylococci which had been grown on horse blood agar had contained immunogenic amounts of equine IgG In parallel absorption experiments with cells grown on media with and without serum it was found that the amount of IgG adsorbed by cells grown on serum containing medium was 85–90 per cent of the amount adsorbed to control cells. Thus means that IgG from the medium blocked only 10–15 per cent of protein A sites on the cells.

DISCUSSION

The protein A content of the extracts of 60 selected strains of *S aureus* was estimated by a modified Mancini technique and by a haemagglutination test. The amount of antigen produced varied from undetectable (less than 2 μg per 10^{10} cells) to 250 μg per 10^{10} cells. The grouping of *S aureus* strains according to protein A production using these methods was essentially similar to the former grouping based on their different affinity to FITC-labelled globulins (Fig 4) However the scattered distribution with regard to protein A production within the FAT groups demonstrates clearly that the FAT offers rough discrimination between the various degrees of protein A production of the pA pos strains.

Forsgren (1) *Sjoquist* (18) and *Sjöquist et al* (20) all used modifications of the Mancini technique (16) for quantitation of protein A from *S aureus* Cowan 1 After growth in broth *Forsgren* determined the amount of protein A in the bacteria to 0.9 per cent (heat extracted protein A) *Sjöquist* to 1 4 per cent (lysostaphin released protein A) and *Sjöquist et al.* to 1 7 per cent (lysozyme released protein A) of the cell dry weight. The data available do not allow a direct comparison of these values with those given in Tables 1 and 2.

For determination of cell wall associated protein A in *S aureus* strains, *Kronvall et al.* (8) measured the uptake of ^{125}I labelled myeloma globulin by 5 × 10^{8} bacteria. The strains Cowan 1 and Cowan 3 were found to form considerable amounts of protein A while Cowan 2 and Wood 46 were protein A negative In a single preparation of a crude extract of Cowan 2 the methods used in this study gave a positive result (53 μg protein A per 10 cells) *Kronvall et al.* noted a pronounced day-to-day variation in protein A production both by Cowan 1 and other

TABLE 2. *Yield of Protein A in Crude Extracts of S. aureus Cowan 1 and 4972 after Growth on Different Solid Media*

Medium	*S. aureus* Cowan 1			*S. aureus* 4972		
	Cells per ml × 10^{10}	μg pA per 10^{10} cells	Yield pA μg per ml	Cells per ml × 10^{10}	μg pA per 10^{10} cells	Yield pA μg per ml
NZ amine type NAK	10.2	135	1377	8.8	193	1968
	11.5	78	897	12.0	209	2508
	4.5	253	1139	–	–	–
Nutrient agar + 10% horse blood	3.4	134	456	6.8	100	680
	5.2	89	463	7.9	120	948
	7.7	86	662	10.0	91	910
	8.1	81	656	12.0	65	780
	4.4	153	673	8.4	100	840
	6.5	110	715	–	–	–
Nutrient agar + 0.1% glucose	6.0	97	582	12.4	90	1116
Nutrient agar + 0.2% $CaCl_2$	5.6	151	846	8.2	69	566
Nutrient agar + 0.2% $CaCl_2$ + 7.5% NaCl	2.0	37	74	5.1	39	199
Phenol red mannitol agar (7.5% NaCl)	2.4	17	41	3.3	33	109

strains. As can be seen from Table 2 this was also the case both for Cowan 1 and 4972 after growth on solid media.

The amount of protein A recovered in the extract of a particular strain is dependent on the medium used for cultivation of the bacteria and of the extraction procedure used. In the present study abundant growth and a high yield of protein A were found after cultivation on solid NZ amine medium. The yield of protein A per 10 cells was significantly lower after growth in broth, presumably because protein A was released from the cells into the medium during the growth period. *Forsgren* (1) found that 30 per cent of protein A formed by Cowan 1 was released into the medium during the exponential phase of the growth and in the beginning of the stationary phase. Later a significantly higher percentage of protein A appeared in the broth. In the present experiments, the yield of protein A obtained from 100 ml broth culture (unwashed cells) after 18 hours growth corresponded to that obtained from the growth on solid medium in six petri dishes (9 cm in diameter). Extraction of protein A by boiling the cells was found to give a low yield of a qualitatively differing protein A preparation, a difference which was also observed by *Sjöquist* (18). As demonstrated previously (9), high concentrations of sodium chloride in the medium suppress protein A formation. For the six strains examined the amount of protein A detected per 10^{10} cells was only 10–20 per cent of that formed on ordinary media. Accordingly *Kronvall et al.* (8) found a 90 per cent reduction in the amount of cell wall associated protein A under similar growth conditions.

The complex formation between protein A and IgG was found to be so intimate that highly purified protein A prepared from staphylococci grown on ordinary horse blood agar contained antigenic determinants that were able to induce antibodies reacting specifically with equine IgG. This selective carrier effect has been studied further with a view to its utilization for production of anti-IgG antibodies (14).

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PRODUCTION OF ANTI-IgG ANTIBODIES BY MEANS OF IgG ADSORBED TO *STAPHYLOCOCCUS AUREUS* COWAN TYPE 1

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Formalin treated cells of the protein A producing strain, *Staphylococcus aureus* Cowan type 1 were suspended in whole serum of the following species man, swine, horse, sheep and cow and then washed thoroughly Immunization of rabbits with these cells, or with cells which had been grown on serum containing medium, gave rise to antibodies reacting specifically with immunoglobulins, above all of the IgG class. Antibodies to human IgG-Fc were obtained when the staphylococci had been suspended in a solution of fragments from papain treated human myeloma IgG's. These antibodies showed no reaction with human IgM or IgA and only minor cross reactions with IgG from other species.

The immunogenic properties of protein A and of protein A producing strains of *Staphylococcus aureus* (*S aureus*) were found previously to be dependent on the presence or absence of serum (IgG) in the medium used for cultivation of the bacteria (4) By immunization of rabbits with staphylococci which had been grown on serum containing medium, antibodies were formed both to staphylococcal antigens and to immunoglobulins due to the complex formation between protein A and IgG (1 2, 6) The utilization of this selective binding ability for the production of antibodies to serum IgG of some mammalian species has been investigated.

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MATERIAL AND METHODS

Bacteria. The *S aureus* strain Cowan type 1 was used because it produces large amounts of protein A when grown on solid medium (4)

Media The basic medium was 4 per cent NZ amine type NAK (Sheffield Chemical) supplemented with 0.0005 per cent thiamine and 0 001 per cent niacin and solidified with 1 per cent Noble Special Agar (4) Three media containing IgG from different species (man, swine, horse) were prepared by adding 5 per cent serum to the basic medium.

Preparation of antigens. After growth for 18 hours at 36 C the bacteria were harvested in phosphate buffered saline at pH 7.2 (PBS) containing 3 per cent formalin, left for ½ hour at room temperature, and washed three times in PBS

A) Cells which had been grown on serum-containing medium were then resuspended in PBS to a density of about 10^9 cells per ml.

B) Cells which had been grown on the basic medium were resuspended to a density of about 2×10^9 cells per ml and divided into portions of 8 ml. Each portion was mixed with 1 ml serum, left for 10 minutes at room temperature,

centrifuged and washed six times in PBS before the suspension was adjusted to a density of 10^9 cells per ml. Sera from the following species were used: man, swine, horse, sheep, cow and rabbit.

C) Cells were treated as described in B) but a portion of only 4 ml of the cell suspension was used and 1 ml of a pool of seven papain treated human myeloma IgG_1 (10) was substituted for the serum. The protein concentration of the papain treated solution containing Fab and Fc fragments was 0.6 per cent.

Immunization procedure. The cell suspension was mixed with an equal volume of Freund's complete adjuvant (Difco). With an interval of 3 weeks, each rabbit received two injections of 1 ml of the mixture subcutaneously. Blood samples were drawn 2 and 6 weeks after the last injection. Rabbits which were immunized with cells grown on serum containing medium received a second booster after a further weeks, and blood samples were drawn 4 and 10 weeks later.

Analytical methods. The rabbit serum samples were analysed against the homologous antigens by immunoelectrophoretic analysis (7) and by crossed immunoelectrophoresis (13). Commercially available rabbit anti-human serum proteins (Dacopatts), rabbit anti-human IgG (heavy chains) (Dacopatts) and swine anti-rabbit serum (Dacopatts and Nordic) were used for comparison. Furthermore, rabbit antisera to serum proteins of other species prepared at Statens Seruminstitut as described in (6), as well as swine anti-human Fc (Nordic) and rabbit antisera to human kappa and lambda chains (Dacopatts) were used for the immunoelectrophoretic analyses. Human serum samples containing Bence-Jones proteins of kappa and lambda types and IgG myeloma protein were included as control antigens.

Precipitation of homologous and heterologous IgG and of staphylococcal protein A by the rabbit sera was studied by double diffusion in agar gel (Ouchterlony analysis) (6).

All rabbit serum samples were tested for staphylococcal antibodies as measured in an indirect haemagglutination test (8), for antibodies to protein A-modified rabbit IgG (5) and for rheumatoid factor by the Waaler Rose test (12).

TABLE 1. *Precipitation of Homologous Antigen (IgG) and Protein A by Sera from Rabbits Immunized with IgG Coated S. aureus Cowan 1 (Ouchterlony analysis)*

Rabbit no.	IgG (species)	Precipitation of	
		Homologous antigen	Protein A
673	Man	+ + +	+
674		+ + +	(+)
675		+ + +	(+)
852	Man (IgG-Fc)	+ + +	0
853		+ + +	+ + +
854		+ + +	+
676	Swine	+ +	+
677		+	0
678		+ + +	+ +
682	Horse	(+)	(+)
683		+ +	+
684		0	+
685	Sheep	+ + +	+ + +
686		+ + +	(+)
687		+ + +	+
688	Cow	+ + +	+ + +
689		+ +	+ +
690		+ + +	0
679	Rabbit	0	+ + +
680		0	+
681		0	+ +
691	*	–	+ + +
692		–	+ +

* *S. aureus* Cowan 1 incubated with PBS instead of serum.

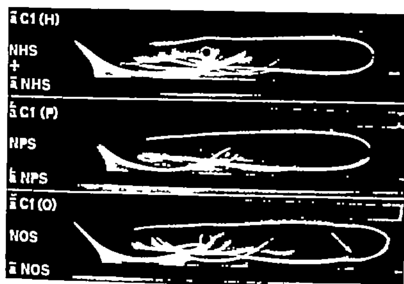


Fig. 1 Immunoelectrophoretic analysis of rabbit antisera to IgG adsorbed to *S. aureus* Cowan 1 (C1). ā C1 (H): rabbit antiserum to C1 coated with human IgG (rabbit 675). NHS: normal human serum. ā NHS: rabbit antiserum to human serum proteins (85, ref. 6). ā C1 (P): rabbit antiserum to C1 coated with porcine IgG (rabbit 676). NPS: normal porcine serum. ā NPS: rabbit antiserum to porcine serum proteins (85, ref. 6). ā C1 (O): rabbit antiserum to C1 coated with ovine slow IgG (rabbit 687). NOS: normal ovine serum. ā NOS: rabbit antiserum to ovine serum proteins (88, ref. 6). The anti-IgG antibodies gave strong precipitation lines with IgG and weak lines with IgM and IgA (just visible on the reproduction). There was no reaction with other serum proteins. The arrow denotes spur due to antibodies specific for ovine fast IgG.

RESULTS

In all groups of rabbits immunized according to the three procedures mentioned under "Material and Methods" the immune response showed pronounced individual variation. In general, an early and strong response to the IgG's of man, sheep and cow could be demonstrated (Table 1).

Serum containing medium as a source of IgG. The amount of IgG adsorbed to staphylococci during growth on serum containing medium is only 10–15 per cent of the amount adsorbed under optimal conditions (4). This fact may explain why the groups of rabbits concerned showed a comparatively low level of precipitating antibodies 6 weeks after the second injection of antigen. The rabbits were therefore given a booster injection. Four and 10 weeks later the anti IgG level was equal to that of commercially available antisera.

Coating of S. aureus *with IgG by incubation with serum.* It has been shown previously by electron microscopic studies and absorption experiments (7–9) that after incubation with serum, protein A producing staphylococci were covered by a uniform dense layer of IgG molecules. Immunization with such cells induced the formation of precipitating anti-Ig antibodies and no antibodies against other serum proteins, thus indicating that these were efficiently removed by the washing procedure. The immunoelectrophoretic analyses revealed strong reactions with IgG (Fig. 1) and in some cases weak reactions with IgA and IgM. In ruminants, the protein A reactivity is limited to the electrophoretically slow IgG (6). As shown in Fig. 1, the antibodies induced against ovine slow IgG reacted with antigenic determinants common for slow and fast IgG but not with those specific for the fast IgG. Detailed analyses of the sera from rabbits immunized with human IgG revealed antibodies precipitating IgG-Fab, IgG-Fc and both types of human light chains.

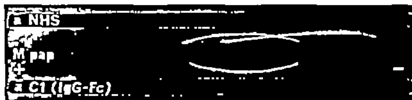


Fig 2 Immunoelectrophoretic analysis of rabbit antiserum to human IgG-Fc adsorbed to *S aureus* Cowan 1 (Cl) a NHS rabbit antiserum to human serum proteins (SS, ref 6) M pap solution of papain treated human myeloma IgG a Cl (IgG-Fc) rabbit antiserum to Cl coated with a pool of seven papain treated human myeloma IgG s. The a Cl (IgG-Fc) reacted only with the Fc fragment of M pap, in which the presence of both Fab and Fc fragments was demonstrated by means of a NHS.

Coating of S. aureus *with human IgG-Fc by incubation with papain treated myeloma IgG* Staphylococcal cells were incubated with papain treated human myeloma IgG in order to obtain anti IgG antibodies that would not react with IgM and IgA. Such cross-reactivity was otherwise induced due to the common light chain determinants of all immunoglobulin classes. As demonstrated for one rabbit in Fig 2, the animals produced antibodies reacting specificially with the Fc fragment of papain treated IgG Reactions with Bence Jones proteins of kappa and lambda types were not found. In Fig 3 the anti IgG Fc obtained is compared with a commercially available anti-IgG (heavy chains) by crossed immunoelectrophoretic analysis. The areas enclosed by the precipitates are approximately equal, which means that the concentrations of precipitating antibody in the reagents compared are similar (13) The anti-human IgG-Fc gave a weak reaction with porcine IgG and no reaction with IgG of the other species studied

Induction of antibodies other than anti-IgG Since IgG was carried on whole cells of *S aureus* antibodies against staphylococcal antigens would be expected to be induced concomitant with antibodies to the heterologous IgG This was the case, as demonstrated in an indirect haemagglutination test a.m. Neter Also in this respect the present groups of rabbits showed a pronounced individual variation of the immune response.

The nature of the protein A reactivity of IgG from the majority of rabbits (Table 1



Fig 3 Comparison of rabbit antiserum to human IgG-Fc adsorbed to *S aureus* Cowan 1 (3A) and rabbit immunoglobulins to human IgG (heavy chains) (Dacopatt) (3B) by crossed immunoelectrophoresis. Both reagents formed only one precipitate when examined with normal human serum as antigen, and the areas enclosed by the precipitates are practically equal in size

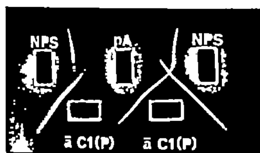


Fig 4 Double diffusion in agar gel (macrotechnique) Upper left and right wells: normal porcine serum diluted 1:100 (NPS) Upper middle well purified protein A 0.1 mg/ml (pA) Lower wells: two rabbit antisera to *S aureus* Cowan 1 coated with porcine IgG during growth on serum containing medium (ā Cl (P)) Both antisera (rabbits 699 and 700) formed a precipitation line with NPS. In addition, one of these (700 right well) formed a precipitation line with pA. This line gave reaction of identity with the line formed between pA and NPS but crossed that formed between ā Cl (P) and NPS (*i.e.*, no reaction of identity)

Fig. 4) was changed from that of forming soluble complexes (3 8) to that of a precipitation reaction as a result of the immunization. This change was accompanied by the occurrence of antibodies to protein A modified rabbit IgG (5) The Waaler Rose test, in which the antigen is rabbit IgG modified through antigen/antibody binding, remained negative in all rabbits.

DISCUSSION

The tempting idea of utilizing the highly specific affinity of staphylococcal protein A for the Fc part of IgG in order to obtain IgG molecules or Fc fragments for immunization was found to be easy to realize. Whether grown on serum containing medium or incubated with serum, the staphylococci carried only the IgG on their surfaces. When human IgG molecules were adsorbed to the bacteria antibodies to IgG-Fc, IgG-Fab and to light chains were demonstrated. The light chain-specific antibodies are presumed to be responsible for the weak precipitates found with IgM and IgA of some animal sera. However when the staphylococci were coated with the Fc fragment of human myeloma IgG, it was possible to obtain antibodies specific for the IgG class of immunoglobulins. For the production of anti IgG reagents, it should be borne in mind that myeloma IgG's are deficient in antigenic determinants (11) and that the reactivity for protein A in some species is limited to subclasses of IgG (3 6)

The antibodies induced against staphylococcal antigens did not interfere with the application of the anti-IgG antisera obtained, at least when methods based on precipitation in gel were used, *i.e* double diffusion in agar gel, single radial immunodiffusion immunoelectrophoresis. The nature of the reactivity between protein A and rabbit IgG was changed during the course of immunization, indicating that the rabbits had produced antibodies against hidden determinants of their own IgG (5) Whether antibodies of this specificity could interfere, for instance, in an immunofluorescence test has not yet been investigated.

The advantage of the immunization procedure described is that only small amounts of material are required and that IgG is obtained in a very simple way

We are indebted to Mrs. *Lene Berthelsen* and Mrs. *Anne Grethe Caspersen* for skilful technical assistance.

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Acta path. microbiol. scand. Sect. B 82: 835–842, 1974

A RAPID METHOD FOR THE DIFFERENTIATION OF *HAEMOPHILUS* STRAINS

The Porphyrin Test

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A rapid and reliable test for demonstrating porphyrin synthesis in *Haemophilus* strains is described. The method is performed with cell suspensions, and tests the ability of the bacteria to use δ-aminolaevulinic acid in the biosynthesis of porphobilinogen and porphyrins. The method permits detection of some of the enzyme activities involved in the haemin biosynthetic pathway and offers as such a more reproducible alternative to the traditional tests for haemin requirement in *Haemophilus* strains. The method described has been applied to 134 *Haemophilus* strains and the results applying to 45 strains from culture collections are listed.

The requirement for the two growth factors nicotinamide adenine dinucleotide (NAD "V factor") and haemin ("X factor") has been important traits in the circumscription of the genus *Haemophilus* and the need for either one or both provide the main means of differentiation of the *Haemophilus* species. The exact determination of these growth factor requirements have unfortunately turned out to be technically difficult with ensuing problems both in identification and classification. This applies particularly to the haemin requirement. The methods have been cultivation on more or less well-defined agar plate media supplemented with the respective growth factors either in discs or directly in the medium. Such methods, besides being time consuming, do not yield unequivocal results if more than one method is used (*Zinnemann* 1960 *Kilian et al.* 1972 *Evans & Smith* 1972)

Studying the biochemical basis of the haemin biosynthetic deficiency *White & Granick* (1963) found that strains of the species *H influenzae H aegyptius* and *H haemoglobinophilus* (canis) all miss the enzymatic capacities to convert δ-aminolaevulinic acid (ALA) to protoporphyrin. Specifically porphobilinogen synthase, uroporphyrinogen I synthase and uroporphyrinogen decarboxylase were found to be missing or inactive in such strains, which accounts for their dependence on haemin for growth. Neither of the intermediate ALA, porphobilinogen (PBG) or coproporphyrinogen of the haemin biosynthetic pathway (Fig 1) permitted growth of these species.

Biberstein et al. (1963) studied the action of *Haemophilus* strains on ALA, and found

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TABLE 1 *Porphyrin Synthesis of Named* Haemophilus *Strains as Revealed by the Porphyrin Test*

Source and No.	Designation from source	Utilization of ALA	Suggested change of classification
NCTC 8502	*H aegyptius*	—	
NCTC 8134	*H aegyptius*	—	
NCTC 8135	*H aegyptius*	—	
NCTC 5886	*H aphrophilus*	+	
NCTC 5906	*H aphrophilus*	+	
NCTC 5907	*H aphrophilus*	+	
NCTC 5908	*H aphrophilus*	+	
NCTC 1659	*H canis*	—	
NCTC 8540	*H canis*	—	
NCTC 10619	*H canis*	—	
ATCC 19416	*H canis*	—	
NCTC 3438	*H gallinarum*	+	*H paragallinarum*
NCTC 8479	*H haemolyticus*	+	*H parahaemolyticus*
NCTC 10659	*H haemolyticus*	—	
NCTC 4560	*H influenzae*	—	
NCTC 4842	*H influenzae*	—	
NCTC 8143	*H influenzae*	—	
NCTC 8465	*H influenzae* (type a)	—	
NCTC 8466	*H influenzae* (type [illegible])	—	
NCTC 7279	*H influenzae* (type b)	—	
NCTC 8467	*H influenzae* (type b)	—	
NCTC 8468	*H influenzae* (type b)	—	
NCTC 8469	*H influenzae* (type c)	—	
NCTC 8470	*H influenzae* (type d)	—	
NCTC 10479	*H influenzae* (type e [illegible] e_3)	—	
NCTC 8455	*H influenzae* (type e_2)	—	
NCTC 8472	*H influenzae* (type e_1 e_2)	—	
NCTC 7918	*H influenzae* (type f)	—	
NCTC 8473	*H influenzae* (type f)	—	
NCTC 10794	*H parahaemolyticus*	+	
NCTC 4101	*H parainfluenzae*	+	
NCTC 7857	*H parainfluenzae*	+	
NCTC 10665	*H parainfluenzae*	+	
NCTC 10670	*H paraphrohaemolyticus*	+	
NCTC 10671	*H paraphrohaemolyticus*	+	
NCTC 10672	*H paraphrohaemolyticus*	+	
NCTC 10556	*H paraphrophilus*	+	
NCTC 10557	*H paraphrophilus*	+	
NCTC 10558	*H paraphrophilus*	+	
ATCC 10801	*H piscium*	+	
NCTC 4557	*H suis*	+	*H parasuis*
NCTC 6359	*H suis*	+	*H parasuis*
NCTC 7440	*H suis*	+	*H parasuis*
NCTC 7441	*H suis*	+	*H parasuis*
NCTC 10535	*H species*	—	

Abbreviation ATCC American Type Culture Collection, Rockville Md.
NCTC National Collection of Type Cultures, London.
ALA δ-Aminolaevulinic acid.

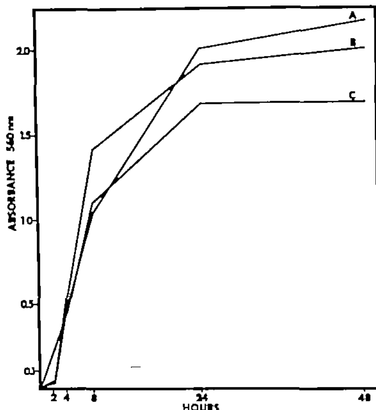


Fig 2 Pattern of PBG synthesis as revealed from absorbance 560 nm of PBG colour reaction in relation to incubation time of three different strains (A: NCTC 7837 2 06 mg dry wt/ml B NCTC 4101 2.49 mg dry wt/ml C NCTC 10671 1.83 mg dry wt/ml) Limit of reaction visible to the naked eye is indicated by a dotted line.

The amount of PBG and porphyrins synthesized during incubation was dependent on the density of the bacterial suspension and the incubation time. These correlations are illustrated in Figs. 2 and 3 and Table 2. As seen from Fig 2 showing the pattern of PBG synthesis, as measured from the PBG colour reaction in relation to incubation time of three different strains, the main increase in colour was seen within the first 24 hours. The values in Fig 2 are the activities to be seen if there is about 2 mg dry weight bacteria

TABLE 2. *Pattern of Porphyrin Synthesis as Revealed from the Degree of Fluorescence in Relation to Incubation Time and Inoculum*

	Undil.*	Dilution of inoculum					
		1 2	1 4	1:8	1 16	1 32	1:64
1 h	—	—	—	—	—	—	—
2 h	+ +	+ +	+ +	—	—	—	—
4 h	+ + +	+ + +	+ +	+	—	—	—
8 h	+ + +	+ + +	+ +	+ +	+	+	—
24 h	+ + +	+ +	+ + +	+ + +	+ + +	+	—

Undiluted One loopfull (about 1 mg dry weight) per 0.5 ml porphyrin test fluid.
— No visible reaction.
\+ Weak red fluorescence
\+ + Definite positive reaction
\+ + + Very strong reaction.

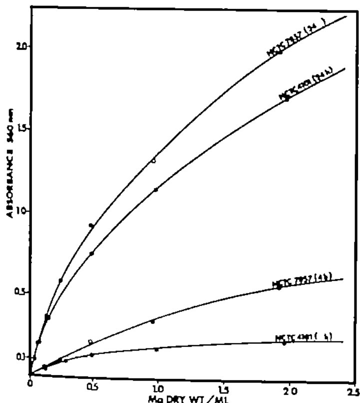


Fig 3 Synthesis of PBG as measured from absorbance 560 nm of PBG colour reaction in relation to inoculum measured as mg dry weight per ml porphyrin test fluid. The pattern of two different strains after incubation for 4 and 24 hours is illustrated. Limit of reaction visible to the naked eye is indicated by a dotted line.

per ml porphyrin test fluid. Using this inoculum (corresponding to one loopfull bacterial cells per 0.5 ml as used in the standard procedure) the reaction of PBG would be clearly visible to the naked eye (limit indicated by the dotted line) after incubation for four hours. The same pattern also applies to the reaction of porphyrins (Table 2) although the fluorescence due to porphyrins normally was visible prior to a positive reaction of PBG. The intensity of the reaction of porphyrins and PBG respectively in relation to size of inoculum is illustrated in Table 2 and Fig. 3 showing that a decreased inoculum required a prolonged incubation to obtain a reaction visible to the naked eye.

Determination of the haemin requirement of the 134 strains by several transfers on two defined agar plate media revealed 58 strains independent of haemin. A comparison between these results and the results of the porphyrin test, showed in the total strain material an 81 per cent positive correlation between independence from haemin and the ability to convert ALA to porphyrins and *vice versa.*

DISCUSSION

The ability to convert ALA to porphyrins was indicative of independence of haemin in the study by *Biberstein et al.* (1963). Conversely inability to carry out these reactions implied, in the 37 strains examined, a haemin requirement. The present study did not show a similar perfect correlation between the results obtained from the porphyrin test and from the method using growth on two defined agar plate media. The latter method has proved superior to a disc method (*Kilian et al.* 1972) but still has the pitfalls of most growth experiments, i.e. the problems of evaluating whether or not growth has taken place. This is stressed by the fact that a 100 per cent reproducibility of the results is not always obtained. The method is further

more dependent on several irrelevant factors that might influence the results obtained, for instance the atmosphere used for incubation of the agar plates. Although *H influenzae* apparently requires haemin for growth under any conditions, there is a substantial reduction in this requirement under anaerobic incubation (*Gilder & Granick* 1947) and strains might consequently be erroneously recorded as haemin independent. Conversely the inability of the basic synthetic medium to meet all growth requirements of occasional strains (*Kilian et al* 1972) might account for a false recording of some strains as haemin requiring.

The porphyrin test described is rapid and reveals some of the actual enzymes involved in the biosynthesis of haemin. The method is furthermore reliable as indicated by a 100 per cent reproducibility. The demonstration of PBG or porphyrins, neither of which are able to permit growth of haemin requiring strains (*White & Granick* 1963) however does not exclude the possibility that strains exist that synthesize these intermediates, but are haemin requiring due to a lack of enzymes later in the haemin biosynthetic pathway. This might also account for some of the lacking correlations of the two methods. The study by *White & Granick* in fact indicates possible differences in the number of enzymes missing in haemin requiring strains. Whereas strains of *H influenzae* and *H haemoglobinophilus* (canis) could form the haemin of cytochromes from protoporphyrin, a strain of *H aegyptius* was unable to form haemin from this precursor apparently missing the ferrochelatase responsible for the incorporation of iron in the haemin molecule (*Porra & Jones* 1963). Neither of the strains, however produced PBG or porphyrins from ALA and would consequently all give negative results in the porphyrin test.

The present study leaves two possible ways of reading the result of the test. The two methods detect two different intermediates in the haemin biosynthetic pathway one of them (PBG) several biosynthetic [illegible] to the other (porphyrin). The tests [illegible]

two intermediates were, however simultaneously positive or negative in all 134 strains examined as they have been in several hundred other strains of *Haemophilus* studied later.

PBG was detected with Kovács reagent which is also used to detect indole formation in many bacteriological laboratories (*Cowan & Steel* 1965). This reagent is indicative of pyrrole rings and is as such not specific for PBG but the present test system is simple and leaves no possibilities of false positive reactions. The red colour due to PBG-p-dimethylaminobenzaldehyde complex is furthermore present in the lower water phase whereas a reaction for indole would be seen in the alcoholic phase. Ehrlich's reagent (*Cowan & Steel* 1965) originally devised for the same purpose, was found to be less satisfactory in the present test, probably because the alcohol component of this reagent, ethanol, is miscible with water in contrast to amylalcohol in Kovács reagent, and therefore dilute the red colour of the PBG reaction.

The PBG reaction is highly dependent on the size of inoculum and on the time used for incubation. One loopfull of bacteria suspended in 0.5 ml of the test fluid normally permitted detection of PBG after incubation for four hours. Differences between strains, however exist and a loopfull is by no means a reproducible unit. Since the addition of Kovács' reagent to the test tube renders a re incubation impossible the test for PBG should not be done too early i.e. normally not before incubation for 24 hours.

When exposed to near ultraviolet light, fluorescence due to porphyrins was normally present from the tubes before a positive PBG reaction could be detected. This is probably due to a much higher sensitivity of the former method (*Schwartz et al.* 1960). This method of reading furthermore has the advantage of leaving open the possibility of repeated readings.

The strains able to metabolize ALA (Table 1) include the named strains of *H parainfluenzae H parahaemolyticus H paraphrophilus H paraphrohaemolyticus H aphro-*

philus, *H gallinarum* and *H suis*. The haemin requirement of the three latter species has been the subject of discussion. In his original description of the species, *Khairat* (1940) described *H aphrophilus* as haemin requiring. This has later been supported by *Boyce et al.* (1968) whereas *King & Tatum* (1962) and *Sutter & Finegold* (1970) claimed their isolates of the species to be independent of haemin. This latter point of view is confirmed by the fact that *White & Granick* (1963) in one of Khairat's strains (NCTC 5886) demonstrated the enzymes of the haemin biosynthetic pathway which obviously also were responsible for the positive reactions of PBG and porphyrins found in the strains of *H aphrophilus* in the present study. Being independent of both of the two growth factors NAD and haemin, the taxonomic situation of this species has to be reconsidered.

The two species, *H suis* and *H gallinarum* also described as haemin requiring (*Lewis & Shope* 1931 and *Delaplane et al.* 1938) raises similar problems which have been discussed by *Biberstein & White* (1969). All strains of these species included in the present study were independent of haemin, and should according to the proposal of *Biberstein & White* be reclassified as *H parasuis* and *H paragallinarum* respectively. The problem remains whether any strains with the characteristics originally described in fact exist.

A strain received as *H haemolyticus* (NCTC 8479) and proposed as working type for this species (*Sneath & Skerman* 1966) was found to be independent of haemin. This is consistent with the findings by others (*Biberstein & Zinnemann* 1971) and the strain should consequently be reclassified as *H parahaemolyticus*.

The haemin requirement together with dependence on NAD and a few other phenotypic traits has played a central role in the classification and identification of bacteria belonging to the genus *Haemophilus*. The reliance on these few characteristics almost to the exclusion of all other characters has obviously hampered the development of a satisfactory classification of this genus. The present study describes a simple and reliable test for demonstration of porphyrin synthesis in haemophili. To equate the results of a positive test with haemin independence is according to the results possible in most cases. The test has, as a part of a taxonomic study been applied to several hundred *Haemophilus* strains with great advantage. The property revealed by the test correlates well with several independent phenotypic traits, and is probably the best single character for the primary subdivision of the genus *Haemophilus* (*Kilian* 1973).

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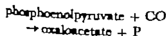
ACTIVITIES OF SOME ENZYMES CONCERNING PYRUVATE METABOLISM IN *NEISSERIA*

ERIK HOLTEN and KAARE JYSSUM

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Phosphoenolpyruvate carboxylase, lactate dehydrogenase, malic enzyme and aspartate aminotransferase activities were found in all strains of selected *Neisseria* species. Aspartase was found in all strains of the "true neisserias" except two, one *N meningitidis* and one *N flavescens* but could not be demonstrated in the "false neisserias. Some species differences in cofactor requirement was noted. Malate dehydrogenase was not found in *N meningitidis*, *N gonorrhoeae* except in one strain which exhibited a trace of activity or in *N cinerea*. In the other *Neisseria* strains, this enzyme was present, but the activities differed markedly between the species.

In an investigation on the enzymes of the tricarboxylic acid cycle in cell free extracts from *Neisseria meningitidis* *Jyssum* (7) demonstrated activities corresponding to lactate dehydrogenase and malic enzyme, among others. Malate dehydrogenase could not be found in this species. As meningococci are able to oxidize acetate (8) alternative pathways of oxaloacetate synthesis would be required if the malate dehydrogenase is inactive also *in vivo* *Jyssum & Jyssum* (9) could demonstrate the formation of oxaloacetate from phosphoenolpyruvate by a phosphoenolpyruvate carboxylase catalyzing the reaction



$$\text{phosphoenolpyruvate} + CO_2 \rightarrow \text{oxaloacetate} + P_i$$

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The formation of aspartate from fumarate, followed by transamination, might provide a by pass around an inactive malate dehydrogenase. Transaminase activity was found in *N meningitidis* (6) but apparently no aspartase activity was present (7) However later investigations have indicated that this species indeed has an aspartase (*Jyssum & Jones* unpublished results)

In the present paper the activities of phosphoenolpyruvate carboxylase (orthophosphate:oxaloacetate carboxy lyase (phosphorylating) E C. 4 1 1.31) lactate dehydrogenase (l-lactate.NAD oxidoreductase, E. C. 1 1 1.27) malate dehydrogenase (l-malate NAD oxidoreductase, E. C. 1 1 1.37) malic enzyme (l-malate.NADP oxidoreductase (oxaloacetate-decarboxylating) E. C. 1 1 1 40) and aspartase (l-aspartate ammonia lyase, E. C. 4.3 1 1) in different *Neisseria* species are compared, in order to collect more data for the investigation of the comparative biochemistry in this genus (2, 3 4 5) All strains have also been screened for aspartate amino-

transferase activity (L-aspartate 2-oxoglutarate aminotransferase, E. C. 2.6 1 1)

MATERIALS AND METHODS

The *Neisseria* strains, media and extraction procedure were the same as those used in previous investigations (2, 5) In one experiment, the minimal medium developed by *Jyssum* (6) was used.

Enzyme assays. In the assay for *lactate dehydrogenase* (7) the reaction mixture contained KCN 3 μmoles, nicotinamide 1.2 μmoles, potassium lactate 10 μmoles, NAD 0.25 μmoles, extract 0.1 ml, *containing 15–25 mg protein per ml, and glycine/* NaOH buffer pH 10.0 250 μmoles, in a total volume of 2.7 ml. In the reverse reaction, 10 μmoles sodium pyruvate was used as substrate and 0.25 μmoles NADH as coenzyme, in 110 μmoles of Tris/HCl buffer pH 7.4

The assay mixture for *malate dehydrogenase* (7) contained $MgCl_2$ 2.5 μmoles, KCN 3 μmoles, potassium malate 10 μmoles, NAD 0.25 μmoles, extract 0.1 ml and Tris/HCl buffer pH 7.4 110 μmoles in a volume of 2.5 ml. The reverse reaction used 10 μmoles potassium oxaloacetate and 0.25 μmoles NADH as substrate and coenzyme, respectively

The assay for *malic enzyme* contained the same reagents as in the malate dehydrogenase assay except that NADP was used as coenzyme.

The reaction mixture for *phosphoenolpyruvate carboxylase* (9) contained $MgCl_2$ 2.5 μmoles, KCN 3 μmoles, sodium phosphoenolpyruvate 2 μmoles, NADH 0.25 μmoles, malate dehydrogenase 12.5 units, extract 0.2 ml and Tris/HCl buffer pH 7.4 100 μmoles in a volume of 2.5 ml.

Aspartase was assayed according to *Dep & Mont* (1) in a mixture containing $MnSO_4$, $MgCl_2$ or $CoCl_2$ 0.5 μmoles, potassium aspartate 37.5 μmoles, extract 0.1 ml and Tris/HCl buffer pH 8.0 47.5 μmoles in a volume of 1.25 ml. After incubation at 37 C for 30 minutes, aliquots were deproteinized with 25 per cent (w/v) trichloracetic acid and analysed for ammonia by direct nesslerization (11) The *reverse reaction was tested in a* mixture containing $MnSO_4$ 0.1 μmoles, NH_4Cl 6 μmoles, potassium fumarate-2,3-^{14}C 7.5 μmoles (specific activity 172,000 cpm/μmole) extract 25 μl and Tris/HCl buffer pH 8.0 9 μmoles in a volume of 250 μl. After incubation at 37 C for 30 minutes, protein was precipitated with 1 volume of 96 per cent (v/v) ethanol and 0.1 volume of 0.1 M EDTA. Aliquots were spotted on Eastman Chromagram cellulose TLC sheets, chromatographed in n-butanol-acetic acid-water (100 2 50) and developed with ninhydrin The spots corresponding to aspartate were transferred [illegible] vials and counted in a Packard Tri-Carb [illegible] tion spectrometer

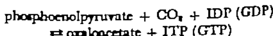
Aspartate aminotransferase was assayed by incubating potassium oxaloacetate 10 μmoles, sodium glutamate 10 μmoles, extract 0.1 ml and Tris/HCl buffer pH 7.4 50 μmoles in a volume of 1.2 ml at 37 C for 30 minutes. The reaction was stopped by heating the vessels up to 100 C for 5 minutes and aliquots were tested for the presence of α-ketoglutarate by glutamate dehydrogenase. In all experiments, appropriate blanks were included.

Enzyme activities are expressed as nmoles of coenzyme reduced or oxidized per minute per mg protein for the NAD- and NADP-coupled reactions, or as μmoles ammonia or aspartate formed in 30 minutes per mg protein for aspartase.

Chemicals. Oxaloacetic and aspartic acids were obtained from Koch-Light Laboratories, Ltd., Colnbrook, Buckinghamshire, England, and malic and fumaric acids from Fluka AG Buchs SG, Switzerland. Sodium pyruvate, barium phosphoenolpyruvate, pig heart malate dehydrogenase and bovine liver glutamate dehydrogenase were purchased from Sigma Chemical Company St. Louis, Mo., U.S.A. Fumaric 2,3-^{14}C acid was produced by The Radiochemical Centre, Amersham, Buckinghamshire, England. Other chemicals were those used previously (5)

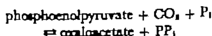
RESULTS

In addition to the phosphoenolpyruvate carboxylase described by *Jyssum & Jyssum* (9) other enzymes are known which carboxylate phosphoenolpyruvate, catalyzing the reactions



phosphoenolpyruvate + CO_2 + IDP (GDP) ⇄ oxaloacetate + ITP (GTP)

(E. C. 4 1 1.32) and



phosphoenolpyruvate + CO_2 + P_i ⇄ oxaloacetate + PP_i

(E. C. 4 1 1.38) These enzymes have not been found in *N meningitidis* (9) In spite of their absence from this species they might well be found in other *Neisseriae* In the present investigation, however only activities corresponding to the phosphoenolpyruvate carboxylase described by *Jyssum & Jyssum* have been examined. Such activity was found in all strains of *Neisseria* (Table 1)

Activities corresponding to lactate dehydrogenase and malic enzyme were also detected in all strains (Table 1)

TABLE 1. *Activities of Phosphoenolpyruvate Carboxylase Lactate Dehydrogenase Malate Dehydrogenase Malic Enzyme and Aspartase in Neisseria*

Species	Phosphoenolpyruvate carboxylase	Lactate dehydrogenase		Malate dehydrogenase		Malic enzyme	Aspartase	
		NAD	NADH	NAD	NADH		Aspartate	Fumarate
N meningitidis								
M 1	14.1	25.9	82.8	0	0	43.7	3 4	0.6
M 5	14 6	27.0	142.6	0	0	37.2	3 7	0.7
M 6	5 2	4.8	4 1	0	0	19.3	2.6	0.7
B 0152/66	9.8	2.3	39 7	0	0	39.5	0.03	0.01
Ne 13	4 0	13 6	31 1	0	0	29.3	3.2	0.7
ATCC 13113	4.4	7 4	367.8	0	0	44 4	3.2	0.3
P 5	12.7	13.2	27.3	0	0	17.0	2.8	0.1
P 17	8.2	22.4	43 4	0	0	28.0	3 7	0.7
P 19	9.5	59.8	143.8	0	0	34.9	1.3	0 1
P 22	6.1	17 1	8.8	0	0	13.5	2.8	0.6
1535	5.3	23.2	197 6	0	0	9.8	3.3	0 4
1873	6.0	14.3	45.0	0	0	17.0	3.6	0.9
N gonorrhoeae								
1 a	2.7	1.8	31.7	0	trace	16.2	3 1	0.7
2 c	3.2	2.5	7.5	0	0	18.1	3 8	0.6
44341	7 1	7.3	174 7	0	0	10.1	3.8	0.4
21306/70	5.2	6.8	10.3	0	0	13.3	3 1	0.4
21319/70	4.3	5.8	116.7	0	0	14 8	3 1	1.6
21332/70	9.8	3.3	43 4	0	0	9.3	3.9	0.3
N sicca								
CN	4.2	12.8	36.8	8.6	101.8	29.0	6 0	1.5
6	3.8	20.3	27 1	7 9	135.5	34.2	4.8	0.9
8021	13 7	12.4	2 4	1.8	113.2	39 1	5.9	2.4
N mucosa								
M 1	12.4	1 4	1 6	34.9	80 4	32.0	6.1	1 9
M 4	9 4	8.4	11.6	44 7	60 6	24 0	4.2	1.5
M 9	14.0	16.5	11.8	44.0	56.0	26.9	3.5	0 7
N perflava								
ATCC 10555	7.3	7.8	23 4	16.2	120.6	15 7	1 6	0.5
A 2	3.7	6.0	6.0	22.0	127 6	12.6	4.2	1 6
191	7.8	4.6	3 3	21.6	197.9	45.6	8.4	1.6
N flava								
ATCC 14221	7 1	10 7	1.8	5.6	58.3	24.3	7 1	1.9
B	9.3	5.8	3 4	6.4	62.6	27 4	6.1	1 7
X 4	8.3	21.0	4.0	4.3	477.8	0.9	6.3	1.8
N subflava								
ATCC 11076	1.6	3.3	9.8	17 2	155.3	35.5	5.3	1.5
ATCC 19243	6.1	1.5	4.0	7 4	102.8	44.3	4.9	0.9
115	6.4	24 9	2.3	1.6	155.6	62.6	6.5	1.8
N lactamica								
ATCC 23970	10.0	5.0	5.0	13 1	0	37.8	4 4	1.3
1379	4 1	42.5	232.3	7 7	0	18.6	4 1	0.3
161 Bc	6.9	14 8	20 7	18.2	0	26.0	4 7	1.2

TABLE 1 (continued)

Species	Phospho-enol pyruvate carboxylase	Lactate dehydrogenase		Malate dehydrogenase		Malic enzyme	Aspartase	
		NADH	NAD	NAD	NADH		Aspartate	Fumarate
N flavescens								
ATCC 13115	3 0	7.8	2.6	61 0	203 4	26.8	3.0	0.1
ATCC 13117	1 6	51.2	6.0	42.9	114.0	42.3	0.1	0.06
ATCC 13120	7 7	18.5	2.6	48.1	178.1	36.7	3 1	1.6
N cinerea								
165/61	8.2	5.7	2.9	trace	0	46.5	7.0	1.2
137/62	6.0	86.4	27.0	0	0	46 0	3 6	1 4
159/62	6.7	11 0	29 0	trace	0	62.8	6.0	1 1
N elongata								
M 2	1.9	19.5	0 6	0	2333	8.1	4.1	0.8
7823/71	1.8	3 9	1.2	0	3049	2.3	3.9	1.2
8554/71	2.0	14 7	1.0	0	6099	4.0	3.8	0 6
N catarrhalis								
ATCC 8176	7 7	7.3	7.3	2 4	4999	6.4	0 1	0.1
Ne 11	6.5	0.5	3 0	0	8481	33.3	0.04	0 07
13016/62	4 4	0	3.9	0	7946	32.1	0.01	0.1
N ris								
199/55	10.3	8.8	8.3	21.2	10144	12.8	0.06	0.1
37/59	6.7	1.3	5.6	trace	3245	14.0	0.05	0.09
917/60	7 1	1.8	7 7	trace	7891	6.5	0.04	0.1
N caviae								
ATCC 14659	5 2	1.3	3 7	0	3291	14.9	0.09	0.05
NCTC 10293	7 9	0.3	5 4	0	6229	13.5	0 1	0.05

Enzyme activities are expressed as nmoles of coenzyme reduced or oxidized per minute per mg protein for the NAD- and NADH-coupled reactions, as µmoles ammonia formed from aspartate or µmoles aspartate formed from fumarate for aspartase.

All strains of *N meningitidis* lacked malate dehydrogenase activity as previously shown by *Jyssum* in the strains M 1 M 5 and M 6 (7) In *N gonorrhoeae* a trace of activity was noted in one strain if oxaloacetate was used as substrate the other ones were negative. Using malate as substrate, no reaction was found in this species. The other strains of *Neisseria*, except those of *N cinerea* had malate dehydrogenase activity In *N lactamica* activity could be demonstrated only if malate was used as substrate An extremely high activity was found in *N elongata* and in the "false neisserias" if oxaloacetate was used as substrate. When malate was used as substrate no activity was found in *N elongata* and only low activities in some strains of the "false neisserias"

Some extracts, especially from *N flavescens* *N cinerea* and *N elongata* could reduce NAD and in some instances also NADP without any substrate added. This effect was not constantly present. The ability to reduce NADP could be removed by desalting the extracts on a Sephadex G-25 column, while some NAD-reducing effect still remained despite this treatment. The endogenous reduction of NAD was sufficiently active to make

calculations of very low activities of lactate and malate dehydrogenase unreliable.

Significant aspartase activity was found in all strains of "true neisserias" except the non-saccharolytic *N meningitidis* B 8152/66 and *N flavescens* ATCC 13117 (Table 1) In the "false neisserias" no significant activity could be demonstrated.

In *N meningitidis* M 6 the reaction had its pH optimum at pH 8.0. The optimal concentration of Mn^{++} was 0 4 mM. These values were used in all experiments. Mg^{++} could also stimulate aspartase activity in all strains, although less efficiently than Mn^{++} When Co^{++} was used as cofactor no significant activity was found, except in *N elongata* where the activity was almost the same with either of the metal ions.

From *N meningitidis* B 8152/66 a mutant which could produce acid from glucose and maltose was made by streaking onto minimal medium enriched with 1 per cent Heart Infusion Broth. The minimal medium contains glucose as the only source of carbon and energy and ammonium ion as nitrogen source (6) Owing to the Heart Infusion Broth, a faint background growth was obtained while mutants which utilized glucose grew well on this medium. The mutant, like the parent strain, had no aspartase activity It was also able to grow on plain minimal medium to which no Heart Infusion Broth had been added. Extract from strain M 6 grown without glucose had as high aspartase activity as extract from blood agar grown cells.

Aspartate aminotransferase activity was found in all strains.

DISCUSSION

No distinct species differences are seen in phosphoenolpyruvate carboxylase lactate dehydrogenase and malic enzyme

Also in aspartase activity there are no significant species differences, except for *N elongata.* In this species, Mg^{++} and Co^{++} are as effective as Mn^{++} in stimulating enzyme activity The difference between the "true" and the "false neisserias" is clear also in the aspartase, as it has been in other enzyme systems examined previously (2 3 4)

Aspartase is not induced by glucose in *N meningitidis* as its activity is not affected in cells grown without glucose. Its activity is not either physiologically coupled to that of glucokinase since the glucose and maltose positive mutant of strain B 8152/66 still is lacking aspartase activity

The differences in malate dehydrogenase activity are of taxonomical as well as physiological interest. It has been shown (7) that *N meningitidis* contains activities corresponding to all enzymes of the tricarboxylic acid cycle except the malate dehydrogenase. If this enzyme is inactive also *in vivo* oxaloacetate must be furnished from other reactions to keep the cycle working. This may be accomplished by phosphoenolpyruvate carboxylase or by aspartase coupled to aspartate aminotransferase. However extract from *N meningitidis* reacts with antibodies to *N perflava* malate dehydrogenase both in double diffusion and complement fixation tests (*Holten* to be published) The failure to detect malate dehydrogenase activity in meningococci may be due either to its presence as "cross reacting material" or to technical difficulties, but in either case the genetic locus for malate dehydrogenase should be present.

Even in the absence of *in vivo* malate dehydrogenase activity acetate might be expected to be oxidized since the phosphoenolpyruvate carboxylase and aspartase/aminotransferase may provide oxaloacetate. *Jyssum et al.* have shown that glucose is rapidly degraded to acetate, but that the oxidation of the latter is incomplete and proceeds more slowly (8) Respirometric studies using labelled glucose indicate that acetate is oxidized by enzymes of the tricarboxylic acid cycle in meningococci (*Holten* in preparation) Which one of the possible pathways of oxaloacetate synthesis is operating *in vivo* is not known. However the ability of the glucose and maltose positive mutant of strain B 8152/66 to grow if glucose is the only source of energy and carbon im-

plies that the aspartase/aminotransferase route is not essential under these conditions. Further studies are needed on this point.

Considerable differences exist between the activities of the malate dehydrogenase in different *Neisseria* species. Extracts from all species of the "true neisserias" react with antibody to *N. perflava* malate dehydrogenase (*Holten* to be published) while the "false neisserias" do not. The antigenic determinants of the enzyme should thus be similar within the "true neisserias" and rather different from those in *N. catarrhalis*, *N. ovis* and *N. caviae*. The observed variations in activity may be due to differences in the regulation of enzyme synthesis or activity. It has, however, not been examined whether the conditions of assay have been optimal in each case.

It remains to be examined whether the differences in malate dehydrogenase activity are correlated to the activity of the tricarboxylic acid cycle. *Tauber & Russell* (10) found an anaerobic acetate oxidizing system in *N. sicca*, *N. perflava* and *N. catarrhalis*, weak activity in some strains of *N. gonorrhoeae* but no activity in *N. meningitidis*. These results seem to agree well with the data on malate dehydrogenase activity in the present report, but it is not known whether the malate dehydrogenase is essential for the oxidation of acetate under the conditions used by these investigators.

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IMMUNOLOGICAL COMPARISON OF NADP DEPENDENT GLUTAMATE DEHYDROGENASE AND MALATE DEHYDROGENASE IN GENUS *NEISSERIA*

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Quantitative micro complement fixation and double diffusion tests were performed with extracts from selected *Neisseria* species, using antisera against NADP-dependent glutamate dehydrogenase from *N meningitidis* and malate dehydrogenase from *N perflava*. *N catarrhalis* *N ovis* and *N caviae* did not react with any serum. Within the "true neisseriae" no distinct differences in reactivity were noted using *anti*-malate dehydrogenase serum. With *anti*-glutamate dehydrogenase serum these species separated in 4 groups with decreasing similarity to *N meningitidis* I *N meningitidis* *N gonorrhoeae* *N lactamica* *N flavescens* *N cinerea* II *N sicca*, *N perflava*, *N flava*, *N subflava* III *N mucosa* and IV *N elongata*.

Taxonomic work in the genus *Neisseria* has been based upon cultural and biochemical tests (2, 3) DNA base composition (10 18) transformation of streptomycin resistance (7 8, 16, 18) nucleic acid reassociation (9 30 31 47) and gas chromatography (13 15 29 32, 37) By these methods, several species have been established. It has also been possible to distinguish between the "true neisseriae" which are related to the pathogenic organisms *Neisseria meningitidis* and *N gonorrhoeae* and the "false neisseriae" which show close compatibility with some *Moraxella* species. Comparison of selected enzymes related to the catabolism of glucose and to the tricarboxylic acid cycle has confirmed the fundamental difference between the two groups of *Neisseria* (24–28)

The study of amino acid sequence resemblance in proteins by immunological methods may give a more reliable indication of the evolutionary relationships among organisms than the methods mentioned above. Information obtained in this way has proved useful in the classification of bacteria (19 21 34 35 38, 40 43–45) as well as of higher organisms (1 41 46 48) The results from such immunological studies have agreed well with studies of DNA homologies in enterobacteria (19) and *Pseudomonas* (43) and there is good agreement between the results obtained by different bacterial proteins (19)

In the present study immunological relationships within the genus *Neisseria* have been investigated. As antigens were chosen

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the NADP-dependent glutamate dehydrogenase from *N meningitidis* and the malate dehydrogenase from *N perflava*. The NADP dependent glutamate dehydrogenase is a constitutive enzyme present in all *Neisseria* species (24 27) Malate dehydrogenase was included in order to test whether its remarkably different activity among the *Neisseria* species (28) could be parallelled by structural differences, and also to see whether cross-reactive material might be found in species which have no detectable malate dehydrogenase activity

MATERIALS AND METHODS

The *N isseria* strains, media and extraction procedure were the same as those used in previous investigations (24 27)

Enzyme activities. One unit of enzyme activity is the amount of extract which oxidizes 1 µmole of NADH or NADPH per minute at room temperature in the presence of the appropriate substrate. Enzyme activities were measured as described previously (27 28)

Purification of enzymes. NADP-dependent glutamate dehydrogenase was obtained from *N meningitidis* M 6 0.05 M Tris/HCl buffer pH 8.0 was used throughout the procedure. The extract was treated with solid $(NH_4)_2SO_4$ and the precipitate forming between 40 and 60 per cent saturation was dissolved in buffer and heated to 60 C for 3 minutes. The supernatant was desalted on Sephadex G-25, and applied on a DEAE-Sephadex A 25 column, 2.5 × 30 cm. After washing with buffer the material was eluted with 600 ml of a linear gradient of KCl (0–0.5 M) in buffer Fractions of 6 ml were collected and those with peak glutamate dehydrogenase activity were pooled. The enzyme was precipitated with $(NH_4)_2SO_4$, applied on a Sephadex G-200 column 2.5 × 80 cm, and eluted at flow rate of 10 ml/hour Fractions of 3 ml were collected. The peak fractions were run once more through DEAE Sephadex A 25 but no significant increase in purity was obtained. After precipitation with $(NH_4)_2SO_4$ the enzyme was dissolved in buffer and stored at –70 C.

Malate dehydrogenase was prepared from extract of *N perflava* ATCC 10555 The material precipitating between 45 and 60 per cent $(NH_4)_2SO_4$ saturation was dissolved in buffer and chromatographed on Sephadex G 200 2.5 × 80 cm at a flow rate of 10 ml/hour Fractions of 5 ml were collected. The peak fractions were concentrated by

TABLE 1 *Purification of NADP-dependent Glutamate Dehydrogenase from* N meningitidis *M 6*

Step	mg protein	Specific activity (units/min/mg)
Crude extract	3863	0 8
$(NH_4)_2SO_4$, 40–60 % sat.	1030	1 4
Heat treatment, 60 C, 3 min	367	4 1
DEAE-Sephadex A 25	56	14 7
Sephadex G 200	15	39.1
DEAE-Sephadex A 25	6.0	41.0

TABLE 2 *Purification of Malate Dehydrogenase from* N perflava *ATCC 10555*

Step	mg protein	Specific activity (units/min/mg)
Crude extract	2342	0.09
$(NH_4)_2SO_4$, 45–60 % sat.	493	0.33
Sephadex G-200	77	0 97
Affinity chromatography	14.3	2.05

precipitation with $(NH_4)_2SO_4$, dissolved in buffer dialysed against buffer containing 0.1 M KCl, and subjected to affinity chromatography on NAD-Sepharose. The column (1.5 × 27 cm) was washed with buffer containing 0 1 M KCl, and eluted with 120 ml of a linear gradient of NADH (0–1 mM) in the same buffer at a flow rate of 10 ml/hour Fractions of 2 ml were collected, the peak fractions were precipitated with $(NH_4)_2SO_4$, dissolved in buffer and stored at –70 C. The purification steps are summarized in Tables 1 and 2.

Immunization. For each enzyme one rabbit received subcutaneous injections at multiple sites on the back of equal volumes of enzyme preparation and complete Freund's adjuvants. After 4 weeks, each rabbit received 3 weekly injections of the enzyme preparation intravenously One week after the last injection the rabbits were bled on 3 consecutive days. The sera from each rabbit were pooled and stored at –20 C. The immunization doses were 1.5 mg protein (61.5 enzyme units) subcutaneously and 0.75 mg intravenously in the case of glutamate dehydrogenase. For malate dehydrogenase the doses were 3.6 mg (7 4 units) subcutaneously 1.8 mg intravenously

Inhibition of enzyme activity. As to *glutamate dehydrogenase*, 2-fold dilutions of serum were mixed with constant amounts of extract. After 15 minutes at room temperature, enzyme activity in the mixture was measured, using α-ketoglutarate as substrate. The amount of serum giving 50 per cent inhibition of enzyme activity was noted. As regards *malate dehydrogenase*, serum and extracts were mixed as in the case of glutamate dehydrogenase. Relatively large volumes of serum were necessary to obtain inhibition, and an extract-independent oxidation of NADH with oxaloacetate as substrate was disturbing the readings. Heating of the serum up to 56° C for 30 minutes followed by dialysis for 24 hours against the diluent used in the complement fixation test (without bovine serum albumin) did not remove this effect. The inhibition is expressed as per cent residual malate dehydrogenase activity in a mixture of 2 ml serum per enzyme unit, after correction for the serum-dependent oxidation of NADH.

Ouchterlony double diffusion was carried out in 1 per cent agarose in 0.85 per cent saline at room temperature for 24 hours. Purified enzymes and crude extracts were used as antigens.

Quantitative micro complement fixation was performed as described by *Levine & Van Vunakis* (33). Erythrocytes from one and the same sheep were used throughout the study. To correct for day-to-day variations in the system, a homologous reaction was included in each day's experiment. The index of dissimilarity (I.d.) for a given antigen is defined as the factor by which the serum concentration must be raised to obtain a complement fixation curve equal in height to that of the homologous system (48). The number of amino acid sequence differences seem to be proportional to the logarithm of this value, hence the term immunological distance has been introduced, defined as 100 × log I.d. (41).

Reagents and chemicals. NAD-Sepharose was prepared according to *Mosbach et al.* (36). To CNBr-activated Sepharose 4B (20), 6-aminohexanoic acid was coupled to act as spacer and NAD was linked to this spacer by dicyclohexylcarbodiimide. DEAE-Sephadex A 25, Sephadex G-25, Sephadex G-200 and Sepharose 4B were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Other chemicals were those used previously (27, 28). Defibrinated sheep blood was obtained from the animal department, National Institute of Public Health, Oslo, Norway. Haemolysin was kindly provided by Dr. *Jornan Eriksen*. As complement was used fresh guinea pig serum, stored in small volumes at −20° C.

TABLE 3. *Inhibition of NADP-dependent Glutamate Dehydrogenase Activity by Antiserum*

Strain	µl serum per enzyme unit giving 50% inhibition of activity
N. meningitidis M 6	13
N. gonorrhoeae 1	11
N. sicca CN	33
N. perflava ATCC 10555	47
N. lactamica 1379	20
N. flavescens ATCC 13120	>250
N. elongata M 2	>200

TABLE 4. *Inhibition of Malate Dehydrogenase Activity by Antiserum*

Strain	% residual activity after the addition of 2 ml serum per enzyme unit
N. perflava ATCC 10555	17
N. sicca CN	28
N. flavescens ATCC 13120	27

RESULTS

Inhibition of enzyme activity. The glutamate dehydrogenase activity was inhibited by specific antiserum (Table 3). In *N. flavescens* and *N. elongata* the inhibition was distinct, but less than that in the saccharolytic *Neisseria*, and the calculation of the serum dose giving 50 per cent inhibition was not possible.

Only the malate dehydrogenase from *N. perflava*, *N. sicca* and *N. flavescens* was inhibited by antibody to the *N. perflava* enzyme (Table 4). The malate dehydrogenase from *N. elongata* was not affected, nor that from *N. lactamica*. In the latter, activity was rather low and only detectable with NAD and malate as coenzyme and substrate (28). In *N. meningitidis* and *N. gonorrhoeae* malate dehydrogenase activity was still not detected after addition of serum.

Quantitative micro complement fixation. The complement fixation curves obtained if purified enzymes or crude extracts from the homologous strains were used as antigen,

Fig 1 Complement fixation curves obtained with purified enzyme (●) and crude extract (×) as antigen. A *Anti*-glutamate dehydrogenase serum, dilution 1·9,000, enzyme and extract from *N meningitidis* M 6. B: *Anti*-malate dehydrogenase serum, dilution 1 2,100, enzyme and extract from *N perflava* ATCC 10555.

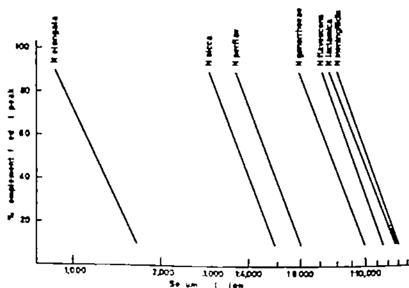


Fig 2 Peak heights of complement fixation curves plotted against the concentration of *anti*-glutamate dehydrogenase serum.

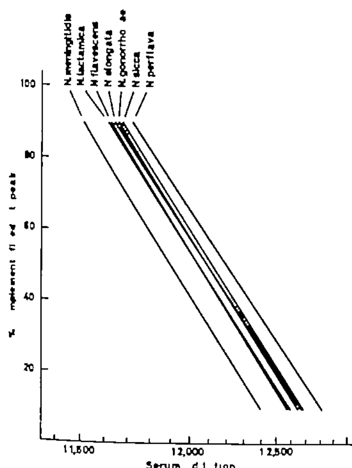


Fig 3 Peak heights of complement fixation curves plotted against the concentration of *anti*-malate dehydrogenase serum.

were identical in shape and height (Fig 1) It is thus probable that the reaction has not been affected by substances other than glutamate dehydrogenase and malate dehydrogenase, respectively and that antibodies to impurities in the enzyme preparations have not been present in amounts sufficient to disturb the reactions.

It has been shown for other proteins that the height of the peak of the complement fixation curve is linearly related to the logarithm of the serum concentration, with heterologous as well as homologous antigen (1 42) For a few strains, peak height was plotted against the logarithm of the serum concentration (Figs. 2 and 3) The curves obtained in heterologous reactions were displaced towards higher concentration of serum, but their slopes were identical. This relationship simplified the calculation of immunological distances.

The immunological distances calculated from the complement fixation tests are recorded in Table 5 The values obtained by *anti*-glutamate dehydrogenase permit the classification of the strains into 4 groups, according to their immunological distances (I.D) from *N meningitidis*

I. I.D 0–15 *N meningitidis N gonorrhoeae N flavescens N lactamica, N cinerea.*
II. I.D 20–40 *N sicca N perflava N flava N subflava.*
III. I.D 70–80 *N mucosa.*
IV I.D 90–105 *N elongata.*

The immunological distance of *N lactamica* ATCC 23970 indicates that this strain

TABLE 3. *Immunological Distances to NADP-dependent Glutamate Dehydrogenase from* N meningitidis *M 6 and Malat D hydrogenase from* N perflava *ATCC 10555*

Species	Strain	Glutamate dehydrogenase	Malate dehydrogenase
N meningitidis	M 1	−0.3	6
	M 3	1.9	7
	M 6	0*	6
	B 8152/66	4	8
	Ne 15	3	3
	ATCC 13113	1.3	1 1
	P 3	−1 1	0
	P 17	−0 4	1.3
	P 19	1.3	1.3
	P 22	0.8	4
	1333	0.2	2
	1875	0	1.9
N gonorrhoea	1 a	11	2
	2 c	13	4
	44341	8	2
	21308/70	3	1 1
	21319/70	9	4
	21332/70	11	?
N mc a	CN	43	1.6
	6	31	6
	8021	23	2
N mu osa	M 1	37	1.6
	M 4	73	−0.3
	M 9	74	−1.9
N perflava	ATCC 10555	34	0*
	A 2	20	2
	191	23	−0.8
N flava	ATCC 14221	36	0.5
	B	37	2
	X 4	32	1.0
N ubfla a	ATCC 11076	30	−0.3
	ATCC 19243	6	3
	115	36	0.7
N lactamica	ATCC 23970	20	3
	1379	1.8	3
	161 8c	−0.2	4
N flav scen	ATCC 13115	200	8
	ATCC 13117	4	8
	ATCC 13170	3	3
N ciner	163-61	4	4
	137 6	0.6	10
	159-62	1 1	6
N longata	M 2	93	
	7823 71	103	0.7
	8554	92	−0.1

TABLE 5 (continued)

Species	Strain	Glutamate dehydrogenase	Malate dehydrogenase
N catarrhalis	ATCC 8176	>210	>130
	Ne 11	>210	>130
	15016/62	>210	>130
N ovis	199/55	>210	>130
	37/59	>210	>130
	917/60	>210	>130
N canis	ATCC 14659	>210	>130
	NCTC 10293	>*10	>130

homologous reaction

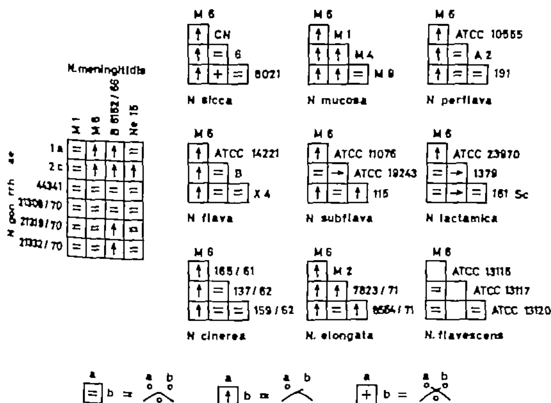


Fig 4 Double diffusion patterns obtained by *anti*-glutamate dehydrogenase serum. Each gonococcal strain is tested against 4 meningococci. Within the other species, all strains are tested against each other and against *N meningitidis* M 6 The symbols indicate reactions of identity (=) partial identity (↑) and double spurs (+)

is not so closely related to the group I strains this is also confirmed by double diffusion (*vide infra*) *N subflava* ATCC 19243 behaves as a group I strain, in contrast to the other strains of this species. *N mucosa* M 1 has only half as great immunological distance as the strains M 4 and M 9 *N flavescens* ATCC 13115 deviates strongly having an

	N meningitidis M6	N lactamica 1379	N flavescens ATCC 13120	N gonorrhoeae Ia	N cinerea 137/62	N perflava ATCC 30555	N subflava ATCC 11076	N flava ATCC 14221	N sicca CN	N mucosa M 4
N lactamica 1379	=									
N flavescens ATCC 13120	=	=								
N gonorrhoeae Ia	=↑	=	↑							
N cinerea 137/62	↑	=	=	↑						
N perflava ATCC 30555	↑	↑	↑	↑	↑					
N subflava ATCC 11076	↑	↑	↑	↑	↑	↑				
N flava ATCC 14221	↑	↑	↑	↑	↑	↑	=			
N. sicca CN	↑	↑	↑	↑	↑	↑	↑	+		
N mucosa M 4	↑	↑	↑	↑	↑	↑	↑	↑	+	
N elongata M 2	↑	↑	↑	↑	↑	↑	↑	↑	↑	+

Fig. 5 Double diffusion patterns obtained by *anti*-glutamate dehydrogenase serum.

immunological distance of 200 The same value was found using an extract made by treating the cells in a Sorvall Omni Mixer with high-speed attachment, operating at maximum speed.

Using *anti*-malate dehydrogenase serum all strains fell within an immunological distance of 10 *N flavescens* *N cinerea* and some strains of *N meningitidis* seemed to have somewhat higher values than the other strains, but the differences were small and no distinct grouping was possible.

No complement was fixed with either serum, using extracts from the "false neisserias" *N catarrhalis* *N ovis* and *N caviae*

Double diffusion The results from the double diffusion tests with *anti*-glutamate dehydrogenase are shown diagrammatically in Fig 4 and Fig. 5 All meningococcal strains showed reactions of identity when tested against each other in all combinations. This was also the case among the gonococci. In interspecies tests, reactions of identity were obtained between strains with an immunological distance from *N meningitidis* M 6 less than 10 A few gonococcal strains with immunological distance greater than 10 showed partial identity to some meningococcal str[illegible] The other strains in group I ex[illegible]pt *N* [illegible] *rea* showed reactio[illegible] of iden[illegible] [illegible] *N* [illegible] *ningitidis* M 6. *N fla*[illegible] ATCC 13[illegible] did not form any precipitat[illegible] [illegible] from group I spurred [illegible] groups II III and IV [illegible] train [illegible] group II spurred over groups III and IV and the groups III and IV both spurred over each other

Using *anti* malate dehydrogenase serum all strains showed pattern of identity with *N perflava* ATCC 10555

Extracts from *N catarrhalis* *N ovis* and *N caviae* did not precipitate with either serum, nor had these extracts influence upon the homologous reactions.

DISCUSSION

From the data obtained by *anti*-glutamate dehydrogenase serum, information can be furnished on the relation between *N meningitidis* and other *Neisseria* species. Relations among the other species cannot be established with certainty Two strains which have similar immunological distances to *N meningitidis* may differ in their glutamate dehydrogenase structure. If they both spur over each other in Ouchterlony tests, each will share some antigenic determinant with *N meningitidis* which the other one does not possess, *cf* *N sicca* CN and *N flava* ATCC 14221 (Table 5 and Fig. 5) This means that experiments with sera against glutamate dehydrogenase from several species would be required to establish complete interspecies relationships.

Within most species there is some heterogeneity *N subflava* ATCC 19243 and *N lactamica* ATCC 23970 have immunological distances like those of group I and II respectively in contrast to the other strains of those species. *N mucosa* M 1 is more closely related to *N meningitidis* than the strains M 4 and M 9 having only half as many amino acid sequence differences as the others. Strain M 1 is by *Berger* considered to be a variety of *N mucosa* called *N mucosa* var *heidelbergensis* (4) This distinction is supported by the present results. *N elongata* 7825/71 is more distantly related to *N meningitidis* than the two other strains. Also by transformation of streptomycin resistance this strain has been found to be slightly deviating (11)

N flavescens ATCC 13115 deviates strong from the others, having an immunological distance of 200. This is probably not due to denaturation of the glutamate dehydrogenase during the extraction procedure, or to protease activity in the extract, since exactly the same immunological distance was obtained with extracts made in different ways. The most plausible explanation is that a mutation has changed the antigenic properties grossly leaving the catalytic activity relatively unchanged.

The near identical reactions with anti malate dehydrogenase serum were somewhat unexpected, bearing in mind the large species differences in the catalytic activity of the malate dehydrogenase (28). Also extracts from species lacking in vitro malate dehydrogenase activity (*N meningitidis* and *N gonorrhoeae*) react with the serum. This must mean that also these species have the locus for malate dehydrogenase. The antibody binding sites are probably located at parts of the molecule which have been well preserved during evolution, possibly the nucleotide binding site. Other parts of the molecule may have changed, causing the great differences in catalytic activity. It is known from tryptophan synthetase in *Escherichia coli* that different parts of an enzyme molecule may evolve at different rates (40).

No reaction takes place with either serum, using extracts from the "false neisserias". According to the relationship between immunological distance and amino acid sequence difference detected in bird lysozymes (39), this would mean that there is more than 40 per cent sequence difference between *N meningitidis* and the "false neisserias". One cannot be sure that the same relationship exists in the present system, but the sequence difference must be considerable. This might also be expected from the fact that the "false neisserias" seem to have only one glutamate dehydrogenase (24).

Also in the present system there is no doubt about the justification of classifying *N elongata* among the "true neisserias". Recent cultural and biochemical tests have also verified this (6), as have transformation studies (11, 12), RNA DNA hybridization (9), gas chromatography (13, 29) and enzymatic investigations using cell free extracts (24–26, 28). Also among other microbes relatively close relationship between coccal and rod-shaped species have been found, although species with different morphology have not been included in the same genus. Using antibodies to D-lactate dehydrogenase, *Gasser & Gasser* (21) found cross-reactivity between *Lactobacillus* and *Leuconostoc* species, and cross-reactivity between *Lactobacillus casei* and *Streptococcus faecalis* was found with antibodies to malic enzyme (35).

Numerical analysis of the fatty acid composition of *Neisseria* and *Moraxella* (29) shows that *N elongata* is similar to *N flavescens*, while *N cinerea* clusters with *N meningitidis* and *N gonorrhoeae*. The clustering of *N elongata* with *N flavescens* is in contrast to the present findings. However, the fatty acid analysis records the product of a number of enzymatic reactions, while the immunological properties of proteins examined in the present study more directly reflects the base sequence of a DNA segment. The results may thus not be directly comparable, as the test systems are entirely different.

One should be careful not to emphasize too much the data obtained by antiserum against glutamate dehydrogenase from only one species. Some conclusions are permitted, however. There are heterogeneities within some species, indicating that the present methods of classifications are not optimal, and that a reclassification of some strains might be questioned. The present findings strongly support the removal of *N catarrhalis*, *N ovis* and *N caviae* from the genus *Neisseria*, placing these species either in *Moraxella* (22) or in a proposed new genus, *Branhamella* (17).

The species *N subflava* and *N flava* react so similar that it might well be justified to unify them in one species, as proposed by *Berger & Brunhoeber* (5).

The species of group I are closely related and may have evolved together until recently

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ISOELECTRIC ANALYSIS OF HAEMOLYSINS AND ENZYMES FROM STREPTOCOCCI OF GROUPS A C AND G

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Crude lyophilised or concentrated culture supernatant fluids from streptococci of Groups A and C were examined in detail by density gradient isoelectric focusing. Two streptolysin O components were consistently detected which had pI values of 6.0/6.1 (major form) and 7.5/7.6 (minor form) respectively. Similar components were observed in Group G material. Two NADases were also revealed with pI values of 8.4 and 8.9 (Group A) and 8.6 and 9.3 (Group C). Esterase activity pI 5.8 was detected in Group C preparations, but not in Group A. Four DNases were resolved with pIs of 4.4 5.8 7.9 and 9.0 the latter two possessing RNase activity. Only the two acidic components were present in the Group C preparations examined. These DNases have been provisionally as C, A, D and B respectively. Streptokinase pI 5.8 (Group A) and 5.4 (Group C) and hyaluronate lyase, pI 4.4 (Group A) and 4.3 (Group C) were also assayed for. Electrofocusing is suitable for quantitative preparation NADase from Group C streptococcal material.

Although much use has been made of the technique of isoelectric focusing for the purification and characterisation of staphylococcal and clostridial toxins and enzymes (2 31) few reports have appeared on similar studies with respect to the many factors elaborated by pathogenic streptococci (1 5 26 32).

These studies were initiated (a [illegible] determine the isoelectric distribution of [illegible] coccal enzymes and haemolysins in [illegible] gradients, (b to evaluate the pot[illegible] technique in the purification of [illegible] antigens of diagnostic value e.g. SL[illegible] tolysin O) DNase B (deoxyribonuclease B) and NADase (nicotinamide adenine dinucleotide glycohydrolase E.C 3.2.2.5) and (c) to further investigate the heterogeneity of SLO (18).

Moreover one of us had proposed on the basis of analytical data, standard criteria of purity and homogeneity and characterisation studies (11 12, 13) that the activities of SLO and NADase were specificities associated with one protein molecule findings recently challenged (1 26) using isoelectric focusing. In a further report (14) it was stated that SLO-NADase preparations possessed esterase activity on a nitrophenyl esters. Thus these relationships were re-examined

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using preparations from both Group A and C streptococci. A brief summary of these findings has been published previously (29)

MATERIALS AND METHODS

Materials Carrier ampholytes, Ampholine® were purchased from LKB-Produkter Stockholm-Bromma, Sweden agarose for enzyme determinations by radial diffusion, from l'Industrie Biologique Française, Gennevilliers, Seine France cysteine hydrochloride, glycine, sucrose, potassium cyanide, acetic acid, imidazole orthophosphoric acid, from Merck AG Darmstadt, W Germany acrylamide and N N methylene bisacrylamide from BDH Chemicals, Ltd., Poole, England hyaluronic acid and hyaluronidase from ovine testes, from Miles-Seravac Ltd., Maidenhead, Berks., England β-mercapto-ethanol, NAD and NADase from *Neurospora crassa* (DPNase) from Sigma Chemical Company St. Louis, USA casein, from N tritional Biochemical Corporation, Cleveland, Ohio, USA; 4-nitrophenyl acetate and DNA from herring sperm, from Schwarz Mann Bioresearch Inc., Orangeburg, USA polyethylene glycol 20,000 and human fibrinogen grade L, (96 per cent clottable, contaminated with plasminogen and FSF Factor XIII) from Kabi AB, Stockholm, Sweden RNA, sodium salt, from yeast, from Koch Light, Colnbrook, Bucks., England α-naphthyl acetate, from Eastman Kodak Co., Rochester N Y., USA fast blue BB salt, from Serva Entwicklungslabor Heidelberg, W Germany DNase, from bovine pancreas, and RNase, from beef pancreas, from Worthington Biochemical Corporation, Freehold, New Jersey USA; streptokinase, Batch no. 583 from Behringwerke AG Marburg Lahn, W Germany Thrombin (Topostasin®) from Hoffmann La Roche, Basel, Switzerland

Bacteria. St ptococcus pyogenes strain C 2035 Lancefield' Group A was kindly supplied by Dr *Walery Hryniewicz* Department of Bacteriology National Institute of Hygiene, Warsaw Streptococcus 22/58, Lancefield' Group G was obtained from the culture collection of Statens Bakteriologiska Laboratorium.

P eparation of concent ated cultur supernatant fluids. Strains C2035 and 22/58 were taken up from freeze-dried ampoules onto 5 per cent (/v) human blood agar plates and incubated anaerobically in GasPak jars at 37 C for 24–48 h Colonies surrounded by good zones of haemolysis were subcultured into 100 ml of pre reduced Brain Heart Infusion Broth (Difco) containing 1 per cent (w/v) maltose (20) These starter cultures were centrifuged at 8,000 × g for 10 min and the sedimented bacteria resuspended in fresh pre-reduced medium. Each preculture served as the inoculum for 1 l of BHI-maltose broth which had been pre-warmed to 37 C. Cultures were performed statically in bottles without pH control and were harvested after 7 to 8 h. Culture supernatant fluids from strains C2035 and 22/58 were desalted using continuous dialysis (33) The dialysed material was concentrated by dialysis against polyethylene glycol 20,000 and then dialysed over night against 1 per cent (w/v) glycine before electrofocusing.

Crude and partially purified lyophilised preparations from streptococci. Freeze-dried streptolysin O (Group A material) was kindly gifted by Dr *Walery Hryniewicz* Department of Bacteriology National Institute of Hygiene, Warsaw It was prepared by the Polish State Vaccine Institute The strain and the culture conditions used were not stated. The preparation bore the markings "Warszawice Streptolyzyna O JM 27 7 serie 1061172 Wazna 27 12.75" This material was dissolved in 0 1 M Tris-HCl buffer pH 7.35 immediately before assays or dialysed against 1 per cent glycine overnight before electrofocusing

Lyophilised supernatant fluid from broth cultures of *St ptococcus* H46A, Lancefield's Group C, was obtained from Behringwerke, Marburg Lahn, Batch N 864/964 Details of the culture conditions were not solicited from Behringwerke. This material contained little salt, ⋜ 10 mM NaCl/g, as measured with a Radiometer conductivity meter CDM3c against standard NaCl solutions and was lectrofocused without prior dialysis.

Preparations of NADase-SLO were partially purified from Behringwerke crude SLO prepared from strain H46A by recycling chromatography on hydroxylapatite (12)

Haemolysin and Enzyme Assays

SLO A haemolytic assay described for θ-haemolysin (28) using twofold dilution titrations in sodium phosphate buffered saline (24) was employed A standardised 1 per cent (v/v) suspension of sheep erythrocytes was prepared as previously described (28) Activation of SLO was performed with neutralised 0 1 M cysteine hydrochloride t a final concentration of 20 mM during the activation procedure by incubation at 37 C for 10 min. The reciprocal of the dilution of haemolysin causing haemolysis of 50 per cent of the erythrocytes in the assay indicated the number of haemolytic units (HU) of SLO/ml (28)

Deoxyribon clease This enzyme was assayed by the radial diffusion method of *S hill & S hamacher* (25) using 1.5 per cent (w/v) agarose plates containing 0.6 per cent (w/v) DNA dissolved in 0.1 M Tris-HCl buffer pH 7.0 made

25 mM with respect to both $MgCl_2$ and $CaCl_2$. Plates were incubated for 18 h at 37 C and developed by flooding with 1 M HCl. Zone diameters were compared with a standard curve prepared with bovine pancreatic DNase included with each set of tests, relating zones of clearing to units of commercial enzyme.

Ribonuclease A radial diffusion assay in substrate-containing agarose was used, 0 4 per cent (w/v) RNA dissolved in 0 M Tris-HCl buffer pH 7.0 made 25 mM with re t o $MgCl_2$ and $CaCl_2$ in 1.5 per cent agarose were developed with 1 M HCl and the z ering compared against a RNase and m beef pancreas.

Both of the nuclease as er rormed with 50 µl samples of fract d ons thereof placed in wells 6 mm d in the gel layers 2.5 mm thick (15 m r to a 9 cm diameter plastic Petri dish

Streptokinase This as r to that developed for staphylokinase dial diffusion was carried out in fibrinogen 0 68 per cent (w/v) fibrinogen diss d n Tris-HCl buffer pH 7.8 containing 0 14 per u (w/v) $CaCl_2$, clotted with 90 N I H uni thrombin. The diameters of zones of liquefaction after 18 h were measured against those ause d by a standard preparation of streptokinase

SH-activated prote c This was assayed by the agarose diffusion method of *I udson* (5) using fractions activ ted with 1 mM β-mercaptoethanol. Incubation was at 37 C for 18 h.

Esterase In essence the assay of *Feh bach* (14) was used, but the 4-nitrophenyl acetate was dissol ed in acetone and stored at 4 C, at which temperature it was stable for several hours. Assays were performed after treatment of test samples with 0 6 mM β-mercaptoethanol for 10 min at 30° C in 0.1 M Tris-HCl buffer pH 8 0 Esterase from hog liver was included as an enzyme control with each set of assays, as were substrate controls for non-enzymic hydrolysis. A Beckman DB-GT recording spectrophotometer coupled to a Beckman Linear Log 10" recorder was used One unit of esterase activity was defined as the amount of enzyme causing an increase in E^{1cm}_{400} = 0 001 per min.

NADase The method of *Cretti & Kaplan* (7) was used with the following modifications The buffer diluent was 0 1 M Tris-HCl buffer pH 7 35 (12) All solutions were kept in an icebath prior to use The substrate was ncubated at 30 C for 3 min prior to addition of enzyme final olume 0.6 ml. All assays were performed in duplicat After incubation for 10 min, tubes were remov to an ice-bath, 3.0 ml of 1 M KCN added the E^{1cm}_{325} of each solution measured after m Appropriate blanks, NAD standard and *N. crassa* NADase controls were included with each set of assays. One unit of NADase activity was defined as the amount of enzyme hydrolysing 1 µmole NAD/min at 30° C and pH 7.35.

Hyaluronate lyase The method of *Smyth & Arbuthnott* was used (28)

Density gradient isoelectric focusing This was performed in principle as described by *McInnes et al.* (23) and *Smyth & Arbuthnott* (28) in broad pH gradients (pH 3 or 3.5 to 10) stabilized in density gradients of sucrose. The anode contained either 0.05 M acetic acid or 0 01 M H PO and the cathode, either 0 09 M imidazole or 0 05 M NaOH. The final potential was between 800 to 1200 V in each case. Columns were drained by insertion of a peristaltic pump in the outlet line or by pumping 65 per cent (w/v) sucrose into the columns and draining from the gradient-loading inlet. Fractions of 1.5 or 2.0 ml were collected. pH measurements were made at 4 C with a Radiometer digital pH meter calibrated with standard pH 6.57 (4 C) buffer (Radiometer Copenhagen). The protein content of fractions was monitored as E^{1cm}_{280} without prior dialysis or by the method of *Lowry et al.* (21) after dialysis.

Gel electrofocusing This was performed as previously described using a constant wattage power supply (30 31) The gel had the composition T = 5 per cent, C = 3 per cent and contained a mixture of Ampholine giving a pH gradient from pH 4 to 6. For esterase zymograms, gels were washed for 5 min in 0 4 M Tris-Maleic acid buffer pH 6.5 at 20 C to remove Ampholine and equilibrate the pH of the gel (4) The esterase bands were located using α-naphthyl acetate as substrate and fast blue BB salt as the coupling agent in 0.1 M Tris-Maleic acid buffer pH 6.4 (22)

Cholesterol inhibition of haemolytic activity This was performed as detailed by *Smyth* (27) for the inhibition of θ-haemolysin.

RESULTS

The elution profile of NADase and SLO from *Streptococcus pyogenes* C203S on electrofocusing is shown in Fig 1A. Two peaks of NADase activity one major and one minor can be seen. Three haemolytic components which were all inhibited by addition of cholesterol were resolved. The most acidic form of SLO was always the major component whilst the most cathodic component was always detected as a minor component. The other minor intermediate form (pI 7.2) was only detected on two occasions with C203S material. Similar experiments with

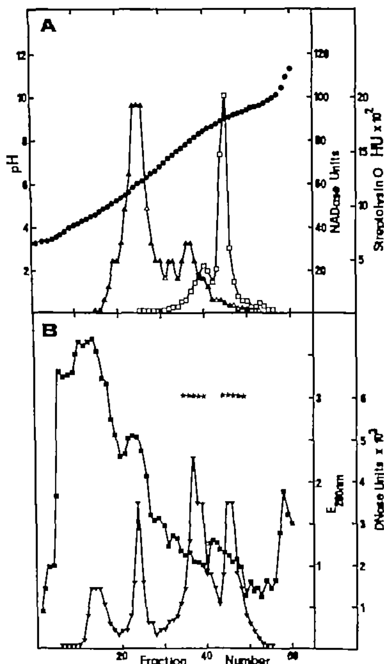


Fig 1 Isoelectric focusing of crude concentrated culture supernatant fluids from *Streptococcus pyogenes* strain C2038, grown in BHI containing 1 per cent maltose. Focusing was performed in broad pH gradient in a 110 ml column (LKB 8101) for 48 h at 4 C to a final potential of 800 V. Anode at bottom of column contained 0.01 M phosphoric acid and the cathode, 0.05 M NaOH. Fraction volume was 2 ml. Activities expressed as units per ml of fractions. The ampholine mixture used comprised pH 3.5–10 and pH 9–11 in the ratio 94 6 (w w)

●●● pH of each fraction t 4 C
▼—▼ DNase activity
★—★ RNase activity
△—△ Streptolysin O activity
□—□ NADase activity
■—■ Protein as OD_{280nm}

the Warsaw SLO preparations revealed the two NADase components, but only one peak of haemolytic activity corresponding to the major form from C203S material. The distributions of NADase and SLO indicate that they are resolvable by isoelectric focusing. The average pIs of these components from five experiments with material originating from Lancefield Group A streptococci are given in Table 1.

Fractions from columns were also screened for streptokinase, DNase, RNase and hyaluronate lyase. The average pIs of these components are given in Table 1. Four DNases were detected as shown in Fig 1B. The two basic forms possessed RNase activity. Esterase and SH activated proteinase were not detected in any of the Group A streptococcal preparations examined.

Electrofocusing of crude Behringwerke Group C streptococcal material gave the elution profiles for NADase, SLO and esterase shown in Fig 2a. The elution profiles of hyaluronate lyase, streptokinase, DNase and protein in the same experiment are shown in Figs. 2b and c. A large band of precipitate formed in the region of the column between pH 4.5–5.5. Precipitate was removed from fractions by centrifugation prior to assays. Two peaks of NADase and SLO were obtained. Again both of the haemolytic components were inhibited by addition of cholesterol. Low haemolytic activity (≷ 40 HU/ml) or none was detectable in peak fractions of the major NADase component. Only two peaks of DNase were found with such preparations which did not possess RNase activity and which corresponded in pI to the acidic DNase components found in Group A material. Hyaluronate lyase and streptokinase gave similar results to the Group A enzymes (Table 1).

TABLE 1 *Isoelectric Points (pI) of Enzymatic and Haemolytic Activities of Streptococci of Groups A and C*

Factor	pI values*	
	Group A	Group C
Streptolysin O	6.1	6.0
	7.6	7.5
	7.2	
NADase	8.9	9.3
	8.4	8.6
Streptokinase	5.8	5.4
Hyaluronate lyase	4.4	4.5
Esterase	–	5.8
DNase (C)	4.4	4.5
(A)	5.8	5.6
(D)	7.9	–
(B)	9.0	–
RNase	7.9	
	9.0	

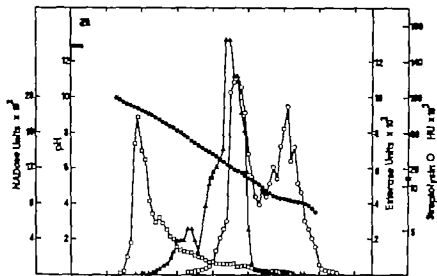
* All pI are average values from at least two experiments.

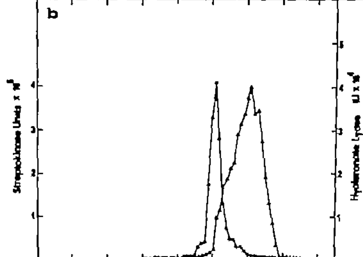
The partially purified Behringwerke preparations were only examined for SLO and NADase and gave similar results to those shown in Fig 2a with the crude material. However only one NADase component corresponding to the major component in Fig 2a was clearly resolved.

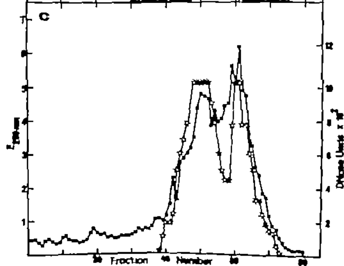
Esterase appeared from the elution profile in Fig 2a to be eluted as two major peaks of activity. One of the peaks overlapped with the major SLO component. Examination of alternate fractions from the elution profile by a zymogram procedure (Fig 3) in combination with gel electrofocusing revealed the

Fig 2 Isoelectric focusing of crude lyophilised culture supernatant from *Streptococcus* H46A of Group C. The material was focused in a broad pH gradient in a 110 ml column at 4 °C for 66 h to a final potential of 1700 V. The anode at the bottom of the column was 0.01 M phosphoric acid, the cathode 0.05 M NaOH. The Ampholine mixture used was pH 3.5–10:pH 9–11 = 94:6. Fractions of 1.5 ml were collected. Activities expressed as units per ml of fraction.

●●● pH gradient
□—□ NADase activity
△—△ SLO activity
○—○ Esterase activity
★—★ Streptokinase activity
▲—▲ Hyaluronidase activity
☆—☆ DNase activity
■—■ Protein as OD_{280nm}


a
NADase Units
pH
Esterase Units
HU


b
Streptokinase Units
Hyaluronate Lyase


c
DNase Units
Fraction Number

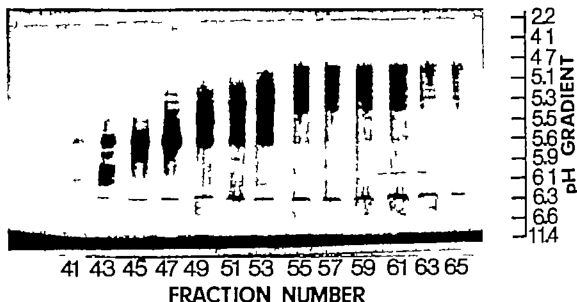


Fig 3 Esterase zymogram in combination with isoelectric focusing in flat bed gel. Electrofocusing was performed at 60 W for 1 h at 4 C to a final voltage of 1600 V The pH gradient was from pH 3.5–10. Esterase bands developed with α-naphthyl acetate/fast blue BB salt in 0.1 M Tris-Maleic acid buffer pH 6.4 Tracks of the gel are numbered to correspond to fraction numbers in Fig. 2.

presence of a major band of esterase activity (pI 5.55) in fractions 43 to 49 corresponding to the more cathodic peak observed in density gradient experiments, whereas the apparent acidic peak comprised 10 to 15 bands. Al though microheterogeneous, the strongest acidic bands appeared in fractions 59–63 (see Fig 3) No SH activated proteinase was found in the starting material.

The average pIs of the factors character ised from Group C material from a total of four experiments are compared with the corresponding results for Group A prepara tions in Table 1

Streptolysin O of low titre prepared from a group G strain 22/58 was also resolved on electrofocusing into two components corre sponding to those identified in Groups A and C preparations. No comparable studies on other factors from Group G strains were per formed.

Data on recoveries and purification of NADase SLO and esterase from the experi ment shown in Fig 2 are given in Tables 2 and 3 Recoveries of NADase were consist ently high with one exception. Moreover pools of two or three peak fractions of NAD ase activity contained some 30–50 per cent of

TABLE 2 *Total Yield f SLO NADase and Est ase Activities after Isoelectric Focusing of Crude Behringwerke H46A Material in a pH Gradient from 3.5 10*

Extracellular product	Total activity applied (units)‡	Total activity recovered (units)‡	Percentage recovery (%)
NADase	2 84 10	2.76 × 10	97
SLO	56 10*	1 79 × 10*	70
Esterase	8 t i 10	2.6 × 10^5	30

‡ Units as defined under M t r l and Methods.
* A erage recover s from seven and fi experiments respectively for NADase and SLO with material from Groups A and C strept re were 85 per cent and 55 per cent respecti ely

TABLE 3. *Purification of SLO NADase and Esterase from Crude Behringwerke H46A Material by Isoelectric Focusing in pH Gradient of pH 3.5–10*

Extracellular factor	Starting material: Activity§ (units/ml)	Starting material: Specific activity $\left(\frac{u/ml}{E_{280}}\right)$	Peak fractions in column: Activity§ (units/ml)	Peak fractions in column: Specific activity $\left(\frac{u/ml}{E_{280}}\right)$	Purification factor (×)
NADase	709	80	1,850	2,840	35
SLO	64,000	7,240	164,000	59,000	8
Esterase	22,150	2,510	10,860	3,320	1 4

§ Units as defined under Materials and Methods.

recovered activity For a single step procedure, the increase in specific activity of NADase was excellent. Pools of peak fractions from type C material were almost completely free of the six other streptococcal factors assayed for (Fig 2)

On the other hand, recoveries of SLO and esterase were reasonable, although insufficient data are available for a critical appraisal of esterase recovery The low degree of purification of esterase may be accounted for by the considerable amounts of precipitate that focused in this pH region of columns with the Group C preparations tested. Similarly SLO focused just to the cathodic side of the bulk of this precipitate.

It is worth pointing out that difficulties were experienced with dialysis of highly purified NADase to remove Ampholine and sucrose. Dialysis against distilled water 0.05M Tris-HCl buffer pH 7 0 and 0 05M sodium phosphate buffer pH 6.8 resulted in 90 per cent inactivation in as little as 5 to 6 h Dialysis against 0 1M Tris-HCl buffer pH 8.5 containing 0 15M KCl and 1mM EDTA appears to be suitable (unpublished result)

TABLE 4 *Comparison of Reported pI Values for SLO*

pI values**						Reference
	≦6 0	*6.5*				(5)
5.8			7 1/7.3§	7.5		(18)
	6.1			7.55		(26)
5.5	*6.0*	*6.5*			8.2	(1)
	6.0/6.1		*7.2*	*7.5/7.6*		***

** Values in italic are those of minor components as designated by authors.
Heterogeneity indicated, details not given.
§ *Alouf J E.*, personal communication to author
*** From this paper

DISCUSSION

In these studies clear resolution of the NADase and SLO derived from streptococci of Groups A and C was achieved, confirming the studies of *Carlson et al* (6) and extending recent studies (1 26) However the presence of two forms of NADase was not previously recorded. pI values of 8.55 (26) and 8.4 (1) for NADase have been reported which could correspond to the minor component observed in the present studies. The difference in pI between our major component and that of these authors cannot be accounted for simply by the temperature dependence of pH which is particularly marked in the basic regions of pH gradients (8) as one group (26) performed their measurements at 4 C (*Grushoff P* personal communication)

Two components were consistently detected in SLO preparations from Group A and C streptococci. Their pIs agree with those reported by *Shany et al.* (26) (Table 4) although the relative distribution of the two components differed. Pronounced microheterogeneity has been observed by two groups (Table 4) but there would appear to be a

consensus for the existence of at least an acidic and a basic form. Although *Alouf* & *Raynaud* (1) demonstrated the existence of a dimeric form of SLO comprising two monomers of 55 000 daltons in material purified by isoelectric focusing it is unclear whether differences in molecular weight contribute to the above heterogeneity

Currently it is not possible to make definitive conclusions on any possible relationship between SLO and esterase from the electrofocusing data. Further experiments are in progress using narrow pH gradients to resolve this question. Although not detected in our studies, Group A streptococci have been reported to produce esterases (19) Thus it is also important to perform comparative experiments with Group A material.

Four streptococcal nucleases termed DNases A, B C and D have been demonstrated previously (35 36 37) They could be separated by starch zone electrophoresis and were immunologically distinct. Moreover both the B and D enzymes possessed ribonuclease activity (35 36) The B enzyme was produced in largest quantity by most strains of Group A streptococci whereas the A enzyme was that produced by the majority of Group C strains (35 36) Also DNase A has been commonly contaminated with streptokinase and DNase B with NADase. Taking these data into account it is possible to provisionally assign the designations shown in Table 1 to the DNases separated by isoelectric focusing. Serological studies with monospecific antisera, heat stability studies and inhibition with bacterial RNA will soon be carried out to confirm this. However the pI 9 0 (DNase B) component was neutralised to high titre by sera from patients who had Group A streptococcal skin infections (*Hederstedt et al* unpublished) Although DNase B has been reported to be [illegible]heterogeneous (17) this was [illegible] observed in the broad pH gradient experiment [illegible]rformed to date

Hyaluronate lyase fro[illegible] [illegible] material gave identical re[illegible] [illegible]ment with the as yet unpublished [illegible] [illegible]rted

TABLE 5 *Comparative Data on the Purification of NADase from Streptococci*

Specific activity§ (units/mg protein)	Purification factor	Recovery (%)	Reference
460	–	–	(6)
2400	10.9	30	(12)
540	2.5	–	(13)
6670	–	c. 85	(26)
6640	35	85	**
9380	25	c. 30	(11)

§ All data converted to units of µmole/min at 37 C between pH 7.3 to 7 4 using $E^{1\%}_{260}$ of 8.0 and a temperature quotient $\left(\frac{\text{activity } 37\ C}{\text{activity } 30^\circ C}\right)$ of 1 87 (unpublished data)
* Calculated from Fig. 2 of the authors.
** From this paper

by *Vesterberg* (32) Whether the differences in the pI values of the Group A and C streptokinases are correlated in some way to the serological varieties reported to be produced by streptococci of these Groups is under further investigation (16) The pI values obtained by isoelectric focusing appear somewhat higher than those recorded by electrophoresis around pH 4 7–5 0 (9 15)

The technique of isoelectric focusing should prove useful in the preparation of NADase, DNase and SLO for diagnostic purposes, but seems particularly suited for quantitative preparation of NADase from Group C streptococcal material. The high pI of this factor allowed its clear separation from the bulk of the protein and precipitate but the use of Group C material also avoided contamination with DNases. Resolution should be maintained by scaling up the procedure to 440 ml columns. Data from previous attempts to purify NADase are shown in Table 5 for comparison. Our preparation is as active as that of *Shany et al.* (26) when the temperature difference of assays is allowed for. The present data on purification and recovery are better with a one-step procedure than *Fehrenbach* (12) obtained with a four step procedure The degree of purification, high spe-

cific activity and quantitative removal of SLO further attest to the usefulness of the technique. Other factors which have not been considered in this report such as optimizing culture conditions and media which are known to be important factors in production, should improve yields.

Further characterization of the distribution of other streptococcal products is necessary for a final assessment of the purity of various factors e.g. SH-activated proteinase streptolysin S lymphocyte mitogen, and erythrogenic toxin (16). Furthermore, possible variations between strains within different groups merit investigation.

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INDIRECT HAEMAGGLUTINATION FOR DEMONSTRATION OF ANTIBODIES TO *ASPERGILLUS FUMIGATUS*

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Subhaemolytic amounts of saline extracts of homogenized, disrupted *A. fumigatus* sensitized tanned erythrocytes for agglutination to high titres (8,192) by an antiserum to *A. fumigatus*. Extracts of 5 different strains of *A. fumigatus* were equally active. Erythrocytes sensitized by similarly prepared extracts of *P. notatum* and an unclassified Aspergillus strain gave low titres (64 and 128) while extracts of some other fungi and bacteria had no sensitizing activity in tests with the antiserum. Results of absorption and inhibition experiments using the various preparations indicated that the sensitizing antigen(s) in extracts of *A. fumigatus* may be species specific. Results obtained with 441 human sera showed good correlation between high titres and presence of aspergillosis. Titres above 128 are highly indicative of active disease.

The diagnosis of human aspergillosis is based on clinical investigations, X rays, repeated demonstrations of Aspergillus (A.) in pathological material, and serological and skin tests (18) with various antigens. However repeated isolations of fungus from sputum is not in itself proof that aspergillosis is present (14) Even antibodies in serum can be of doubtful diagnostic value, the most important clue is increase or decrease in titres.

Various serological tests have therefore been used Complement fixation (1 13 21 22) double diffusion in agar (2 3 4 7 19) latex agglutination (10 20) collodium agglutination (20) and immuno-electrophoresis (1 11 12, 21) Because extracts of *A. fumigatus* haemolyse erythrocytes, it has been claimed that indirect haemagglutination could not be used (9 20) Complement fixation and latex agglutination give too low titres to be of diagnostic value (16 20 21 22) and double diffusion in agar has therefore been most widely employed. The interpretation of results can be confusing however as cross-reactions with similarly prepared antigens from other fungi are frequent (1)

Since clinical and radiological diagnosis of aspergillosis often encounters difficulties, a sensitive and specific serological technique would be an important tool. Efforts were therefore made to elaborate a haemagglutination test which allows an easy semiquantitation of the antibodies. The results are described in the present paper

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MATERIALS AND METHODS

Aspergillus Strains

Most of the experiments were carried out with a strain of *A. fumigatus* isolated in the Department of Microbiology (later called Af) Four strains were obtained from Commonwealth Mycological Institute, Kew Surrey England *A fumigatus* IMI 16030 48338, 131705 and 92877 A strain of Aspergillus isolated in this department and which could not be species determined was included (Aspergillus 3) *A pseudoglaucus* and *A niger* were kindly provided by the Institute of General Microbiology University of Bergen, and the State Institute of Public Health, Oslo respectively

Other Fungi and Bacteria

The following species were included *P notatum C albicans T rubrum T schoenleinii S aureus Micrococcus* strains and *E coli*

Erythrocytes

Whole blood from sheep rabbit, guinea pig and chicken were collected in equal amounts of Alsever s solution, and human blood in acid-citrate-dextrose solution. The blood was kept at 4 C. Before use the erythrocytes were washed 3 times in phosphate buffered saline, pH 7.2 (PBS) with centrifugation at 800 × *g* for 5 min and final packing at 1,000 × *g* for 10 min.

Formalin treated erythrocytes were prepared as described by *Daniel et al.* (5)

Sera

Four groups of human sera were selected as follows 1) randomly selected sera from blood donors (131) 2) sera from patients w th various respiratory diseases (44) 3) sera from patients with various diseases (231) and 4) sera from patients with definite or possible aspergillosis (35)

The patients of the 3 control groups (1–3) were selected to give an age distribution similar to that in the patient group They were not matched according to sex.

Most of the sera from patients with respiratory diseases were received from the Department of Lung Diseases, Bergen University Hospital.

An antiserum to *A fumigatus* Af w raised in a rabbit by immunization w th a lyophilized washed culture suspended in PBS The schedule consisted of weekly injections of 2 ml, the first week given subcutaneously and containing 1 6 mg ml followed by intramuscular injections, and increasing the dose to 20 mg/ml The rabbit recei ed 6 injections and then a booster-dose 3 weeks later Small bleedings were taken before each injection, and larger bleedings 1 week after the 6th and the 7th injections.

Before use all sera were heated at 56 C for 30 min (inactivated sera)

Cultures

Surface culture From pure cultures on Sabouraud s medium (Difco) the fungi were transferred to Roux bottles containing yeast extract medium (15) The bottles were incubated at 37 C until a continuous mat of growth was established on the surface. The necessary incubation time varied from 3 days to 5 weeks, most of the Aspergillus strains had the shortest, and *T schoenleinii* the longest time. *C albicans* was only grown on Sabouraud agar plates and the bacteria on nutrient agar plates.

Suspension culture Fungi were transferred to 1,000 ml Erlenmeyer flasks containing 30 ml yeast extract medium. The flasks were incubated at 37 C on a shaker for 3 days. Material from such cultures contained ery few conidia, as shown by *Fukai & Yasuda* (8)

Harvesting was performed under a hood. The medium in the Roux bottles was poured off and the harvest shaken carefully with 1,000 ml of distilled water the mycelium was transferred to a Buchner funnel and washed with 1,000 ml of distilled water

Harvesting of the suspension culture was performed by separating the mycel clumps from the medium by filtration through filter paper transfer to a Buchner funnel and washing with 2,000 ml of distilled water

The harvests were then homogenized for 4 min at 180,000 rev/min in an Omni Mixer (Sorvall) in ice water-bath with a small amount of PBS, and the suspension was transferred to a bacterial press (X-press, AB Biox, Stockholm) After freezing at –25 C, the material was pressed 3 times. After lyophilization the material was kept in small screw-cap bottles at room temperature.

Preparation of Extracts

The lyophilized material was resuspended in PBS and left at room temperature for 20 min. Unsoluble material was spun down at 1,000 × *g* for 5 min, and the supernatant (extract) was saved. The concentrations of the extracts will be given as mg lyophilized material per ml of PBS.

Titration f Haemolytic Acti ty of the Extracts

To twofold dilutions of extracts in 0.2 ml of PBS was added 0.2 ml of a 2 per cent suspension of erythrocytes After incubation at 37 C for

30 min followed by 90 min at 20 C, the degree of lysis was recorded macroscopically 100 per cent: 3+ 50 per cent 2+ 25 per cent 1+ 0 per cent: –

Indirect Haemagglutination (IHA)

Equal volumes of 2.5 per cent sheep erythrocytes and tannic acid (E. Merck, Darmstadt, W-Germany) diluted 1 in 80,000 (w/) were incubated at 37° C for 10 min. The tanned erythrocytes were washed thrice in PBS and packed. To 10 ml of a 0 5 per cent suspension of tanned erythrocytes was added 0.5 ml of extract at proper concentrations. The mixtures were incubated at 37 C for 60 min. The sensitized erythrocytes (SSB) were washed thrice in PBS containing 2 per cent normal rabbit serum (diluent) and resuspended to 0.5 per cent in diluent.

All sera to be tested were absorbed with sheep erythrocytes by mixing equal amounts of serum diluted 1 in 4 and packed erythrocytes. After 15 min at room temperature, the erythrocytes were sedimented by centrifugation and the supernatant (absorbed serum) was saved.

The haemagglutination test was performed with microequipment (Cooke Engn. Co., Medical Research Division, 900 States Lane, Alexandria, V USA) To twofold dilutions of absorbed serum in 0.025 ml diluent was added 0 025 of SSB. The plates were shaken, incubated at 37 C for 30 min and then overnight at 4 C. The haemagglutination was graded as 3+ 2+ 1+ and – according to the appearance of the pattern of sedimented cell The titre is given as the reciprocal of the highest serum dilution which gav agglutination

Inhibition of IHA

To 0.025 ml of extracts of various concentrations was added 0.025 ml of antiserum to *A fumigatus* Af diluted in diluent. The mixtures were left at room temperature for 45 min upon which 0.025 ml of SSB was added. Patterns were recorded as for IHA.

Absorption of Sera

To 0.5 ml of the antiserum to Aspergillus were added 50 mg of lyophilized fungus material. The mixture was left at room temperature for 60 min and then centrifuged at 1,000 × g for 10 min. The supernatant (absorbed serum) was tested in IHA.

RESULTS

Haemolytic Activity of Extracts

Since it was known from earlier (15 20) that saline extracts of Aspergillus haemolysed erythrocytes, we tested extracts of *A fumigatus* Af against various species erythrocytes to find which erythrocytes were liable to lysis (Table 1) Erythrocytes from man and guinea pig were most sensitive, those from rabbit and chicken were least sensitive, while sheep erythrocytes showed medium sensitivity Since rabbit and chicken erythrocytes are not well suited for indirect haemagglutination with human sera, we selected sheep erythrocytes for further experi-

TABLE 1 *Haemolysis of Different Species Erythrocytes by Extracts of* A. fumigatus *at Various Concentrations*

Erythrocytes from	Amount of material extracted (mg/ml)				
	60	30	15	7.5	3 75
Man	3+	3+	2+	1+	–
Sheep	3+	3+	2+	–	–
Rabbit	3+	3+	–	–	–
Guinea pig	3+	3+	3+	1+	–
Chicken	3+	3+	–	–	–
Man (F)	–	–	–	–	–
Sheep (F)	–	–	–	–	–
Rabbit (F)	–	–	–	–	–
Man (T)	3+	3+	2+	1+	–
Sheep (T)	3+	3+	2+	–	–
Rabbit (T)	3+	3+	1+	–	–

F: Formalinized.
T Tanned.

TABLE 2 *Extracts of Cultured Material from the Various Aspergillus Strains Tested for Haemolysis of Sheep Erythrocytes*

Extract of	Strains	Amount of material extracted (mg/ml)				
		120	60	30	15	7.5
A. fumigatus	Af (Sf)	3+	3+	3+	2+	–
	Af (Sp)	–	–	–	–	–
	16030 (Sf)	3+	3+	3+	2+	–
	16030 (Sp)	–	–	–	–	–
	131705 (Sf)	1+	–	–	–	–
	48338 (Sf)	–	–	–	–	–
	92877 (Sf)	3+	2+	–	–	–
A. pseudoglaucus		3+	3+	1+	–	–
A. niger		–	–	–	–	–
Aspergillus 3		–	–	–	–	–

Sf Surface culture.
Sp Suspension culture.

ments. It should be noted that tanned erythrocytes showed unchanged sensitivity to the haemolysin while formalinized erythrocytes were resistant.

Extracts of the various Aspergillus cultures were then tested for haemolytic activity against sheep erythrocytes. Table 2 shows that the activity varied between strains, and also within strains according to type of culture used. Extracts of material obtained from suspension cultures gave no lysis.

Sensitization of Erythrocytes for IHA

Sheep erythrocytes were treated with extracts at the concentration of 1/2 of the haemolysing amount or at a concentration of 120 mg/ml of non-haemolytic extracts. After washing and resuspension the erythrocytes were added to dilutions of the immune serum. No agglutination occurred, indicating that the extracts did not sensitize the erythrocytes.

Tanned erythrocytes were then treated similarly and tested against the antiserum to Aspergillus (Table 3). Extracts of all 5 strains of *A. fumigatus* sensitized erythrocytes for agglutination to high titres, while extracts of Aspergillus 3 and *P. notatum* gave very low titres. Extracts of the other fungi did not sensitize the erythrocytes. Erythrocytes sensitized by extracts of material from suspension cultures gave usually titres lower by two steps, even with a concentration of 120 mg/ml and the results were inconsistent. Further experiments were therefore performed with extracts of the surface cultures.

Lyophilized preparations kept at room temperature for 12 months showed no decrease in sensitizing activity. However extracts of fresh, homogenized material which had not been disrupted in the bacterial press did not sensitize the erythrocytes.

TABLE 3 *Titres of Indirect Haemagglutination with Rabbit Antiserum to* A. fumigatus *Tested Against Sheep Erythrocytes Sensitized by Extracts of the Various Fungi*

Extracts of	Titres
A. fumigatus (5 strains)	4096 16384
Aspergillus 3	1 8
P. notatum	64
A. niger and *A. pseudoglaucus*	1
C. albicans	1
T. rubrum	1
T. schoenleinii	1

Specificity of the Sensitizing Substances

The specificities involved in agglutination of erythrocytes sensitized with *A. fumigatus*

TABLE 4 *Indirect Haemagglutination with Antiserum Tested Against Erythrocytes Sensitised by Extracts of* A. fumigatus *Af Aspergillus 3 or* P notatum. *Effect of Absorption of Serum with Cultured Material of Each Fungus*

Erythrocytes sensitized with	Antiserum to *A. fumigatus* Af	Serum diluted 1 in 4	16	64	256	1024	4096	16384
A. fumigatus Af	Unabs.	3+	3+	3+	2+	1+	1+	–
	Abs. with							
	A. fumigatus Af	–	–	–	–	–	–	–
	Aspergillus 3	3+	3+	3+	2+	1+	1+	–
	P notatum	3+	3+	3+	1+	1+	1+	
Aspergillus 3	Unabs.	1+	1+	1+	–	–	–	–
	Abs. with							
	A fumigatus Af	1+	1+	1+	–	–	–	–
	Aspergillus 3	–	–	–	–	–	–	–
	P notatum	1+	1+	1+	–	–	–	–
P notatum	Unabs.	1+	1+	1+	–	–	–	–
	Abs. with							
	A fumigatus Af	1+	1+	1+	–	–	–	–
	Aspergillus 3	1+	1+	1+	–	–	–	–
	P notatum	–	–	–	–	–	–	–

extracts were then investigated in inhibition studies using extracts of all fungi and bacteria mentioned (see Materials) against 4 agglutinating units of the antiserum. The results were clear-cut Extracts of all 5 strains of *A. fumigatus* (from 1.25 to 10 mg/ml) inhibited the agglutination of erythrocytes sensitized with extracts of the *A. fumigatus* strains, while concentrations up to 120 mg/ml of the other organisms had no inhibiting effect.

Results of absorption experiments were in perfect accordance with the results of inhibition. Antiserum was absorbed with lyophilized material from various cultures including the 3 species of bacteria, and absorbed and unabsorbed serum samples were tested against erythrocytes sensitized with *A fumigatus* Af. The material from all 5 *A fumigatus* strains absorbed the agglutinins, while none of the other fungal or bacterial preparations had any effect. Particularly Aspergillus 3 and *P notatum* which sensitized erythrocytes for agglutination with antiserum, did not reduce the titre.

The latter results were further supported by the following observations. Samples of antiserum were absorbed with lyophilized material from *A. fumigatus* Aspergillus 3 and *P notatum* respectively The results presented in Table 4 show that each strain of fungi only absorbed the corresponding antibodies. This indicates that the concentration of the cross-reacting antigens are relatively

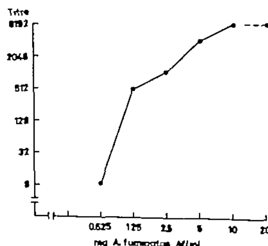


Fig 1 Titres in indirect haemagglutination of rabbit antiserum to *A fumigatus* Af tested against erythrocytes sensitized with various amounts of Aspergillus extract.

TABLE 5 *Indirect Haemagglutination. Human Serum Tested Against Erythrocytes Sensitized with Extract of* A. fumigatus *Af Distribution of Number of Sera According to Titres*

Serum from	No.	Titres <8	8	16	32	64	128	256	512	1024	2048	4096	8196
Blood donors	131	122 (94)	6 (4)	3 (2)									
Patients with lung diseases	44	38 (87)	3 (7)	1 (2)		1 (2)	1 (2)						
various diseases	231	189 (82)	4 (2)	6 (3)	4 ( )	22 (9)	5 (2)		1 (0.5)				
obs. aspergillosis	35	14 (40)		3 (9)		6 (17)	1 (3)	1 (3)	4 (11)	2 (6)	1 (3)	2 (6)	1 (3)

Figures in parentheses Per cent.

low in *A fumigatus* and that the sensitizing antigen is fairly specific for *A fumigatus*

The antiserum was titrated against sheep erythrocytes sensitized with *A fumigatus* Af extract at various concentrations prepared as twofold dilutions from an extract of 20 mg/ml (checkerboard titration) The results are shown in Fig 1 Extract at a concentration of 10 mg/ml sensitized the erythrocytes optimally This sensitization was therefore employed in further experiments. (Higher concentrations lysed the erythrocytes as shown above)

All bleedings from the immunized rabbits were tested in IHA. The titres rose from <2 in the pre-immune samples to 4,096 after the 3rd injection and to 8 192 one week after the booster dose. This indicates that the technique will easily detect changes in titres during immunizations.

Haemagglutinins in Human Sera

The 4 groups of human sera were tested in IHA as developed. Each serum was tested at least twice, and for each test the following control sera were included 1) the antiserum, 2) a high titred serum (titre >512) 3) a medium titred serum (titre 128) and 4) a low titred serum (titre 16) Maximum variation of these control sera was ± 1 dilution. The results are shown in Table 5 which is compiled on the basis of the mode titres for each serum. Among the blood donor sera, as many as 94 per cent had titres <8, and none had titres >16. Among the 275 sera from patients with various lung diseases and other diseases, only one had a titre >128. This was a patient with upper respiratory allergy but the aetiology could not be established. Among the sera obtained from patients under observation for possible aspergillosis, 32 per cent had titres >128. It should be noted that, as regards the 17 patients whose sera had titres <32, the diagnosis aspergillosis was ruled out after the observation period. On the other hand the 4 sera with titres >1,024 were from patients who after evaluation had definite aspergilloma or aspergillosis, while the 6 sera with titres 64 to 1,024 were from patients with either definite or possible aspergillosis or aspergilloma. Serum from a patient who underwent surgery for aspergilloma 6 months earlier had a titre of 512.

Sequential sera were obtained during treatment of two patients with definite aspergillosis (asthma and bronchitis) The sera were kept frozen and tested on the same day Sera from both patients showed a 4- to 8-fold decrease in titres after 3–4 weeks.

DISCUSSION

In accord with earlier reports (15 20) we also found that extracts of surface cultures

of Aspergillus were haemolytic for erythrocytes. The activity varied somewhat with species of erythrocytes, but all were lysed. Since sheep erythrocytes showed medium lysis and are widely used in routine serology we selected these for our experiments.

Untreated erythrocytes were not sensitized by sub-haemolytic amounts of the extracts for agglutination by antiserum to Aspergillus. But tanned erythrocytes were not lysed to a higher degree, and sensitization could therefore be performed with a concentration of extracts which was close to the haemolytic amount. The titres obtained with antiserum tested against such sensitized erythrocytes were in a high range i.e. the test showed a high sensitivity. This was also clearly demonstrated by the sequential sera from the immunized rabbit. The titre increased from <2 to 4,098 in 3 weeks.

The results obtained by comparing the sensitizing activity of haemolytic and non-haemolytic extracts of *A fumigatus* (extracts of surface and suspension cultures, respectively) showed that the sensitivity could not be increased by using concentrated non-haemolytic extracts. These results indicated that the sensitizing activity of each extract reached an optimal level of concentration at sub-haemolytic amounts. By checkerboard titrations of antigen preparations and antisera, or titration of antigen in inhibition tests, the optimal concentration of sensitizing antigen can easily be determined employing another *A fumigatus* antigen preparation.

A possibility which we did not explore further in this series of experiments, was to use *formalinized* erythrocytes as recently reported by *Senat & Brisset* (17). Such erythrocytes were not lysed by the extracts, but in our preliminary experiments they gave inconsistent results in IHA.

The immune serum produced against *A fumigatus* Af agglutinated erythrocytes sensitized by any of the 5 *A. fumigatus* extracts to similar titres, while erythrocytes sensitized by most of the extracts of other fungi, or bacteria, including *A niger* and *pseud glaucus* were not agglutinated. Erythrocytes sensitized with extracts from *P notatum* and Aspergillus 3 gave low titres. Apparently the sensitizing antigen in extracts of *A fumigatus* is species specific. The results of absorption experiments using the lyophilized cultured material of the various organisms supported this observation. Only *A fumigatus* material reduced the titres of the immune serum tested against *A fumigatus* antigen in IHA. However experiments with antisera produced against other Aspergillus species and other fungi, together with extensive absorption experiments, are needed if the specificity of the described reactions is to be definitely established.

A technical point which should be stressed is the importance of complete disruption of the fungus material before preparation of the extract. No sensitizing activity was traced in extracts of material which had not been disrupted in the bacterial press.

The results obtained with the 441 human sera showed good correlation between high titres and presence of aspergillosis, and none of the sera with low titres were from patients with the disease. However we could not establish a definite lower limit suggestive of specific disease. On the basis of the titres recorded we used the following groupings until a larger series of patients and controls had been investigated Titres ≦32 "normal" titres 64–128 doubtful increase, and titres ≧256 pathologically increased. It is highly probable that patients whose sera fall within the last group have aspergillosis. The test may therefore be useful since *A fumigatus* is responsible for 90 per cent of reported cases (6)

The technique presented is simple to perform. The sensitivity of the technique makes it easy to detect increases or decreases in titres while patients are under observation particularly since between 80 and 90 per cent of the sera in the 3 control groups had no demonstrable antibodies (titre <8) Results from this study indicate that the antigen is connected with the mycelium, since conidia free material contained it. Results of experiments designed to characterize the antigen

and to investigate the relationship between haemagglutinating and precipitating antibodies to *A fumigatus* will be presented in a forthcoming paper

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OBSERVATIONS ON THE RELATIONSHIP BETWEEN *ASCARIS SUUM* BURDENS IN PIGS AND FAECAL EGG COUNTS

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Faecal *A. suum* egg counts were carried out on 69 bacon pigs of which 50 harboured *A. suum*. Correlation was found to be poor between the egg counts and (1) the total number of *Ascaris* (2) the weight of the worms, and (3) the ratio between male and female worms. Only 4 out of 50 *Ascaris* infected pigs harboured macroscopically detectable juvenile stages of *A. suum*. It is concluded that the size of *A. suum* burdens is not to be judged from the number of eggs in the faeces except, perhaps, at very low levels of eggs, *i.e.* <30 e.p.g. (eggs per g of faeces) Additionally it is suggested that *A. suum* burdens are not continuously established once the pigs are infected.

Though *Ascaris suum* (*A. suum*) is considered the most injurious parasite in pigs, very little is known about the dynamics of the worm burdens. It is inevitable, in order to cope successfully with this parasite, to increase the knowledge concerning certain aspects of ascariasis by *e.g.* quantitation of worm burdens and patterns of worm replacement. Such an approach to the problems has been recommended by an Expert Committee (WHO) in 1967 as regards human ascariasis. Similarly *Michel* (1963) stressed that the understanding of the factors regulating the course and size of nematode infections is fundamental to any effors towards a control of the infections.

The present investigation was devised with a view to contributing to the elucidation of the dynamics of porcine ascariasis. The main emphasis was put on the relationship between faecal egg counts and the number of worms, the weight of worms, and the ratio between worms.

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MATERIALS AND METHODS

Animals. The entire stomach and intestine from 69 bacon pigs were collected at two slaughter houses. They were collected in order to ensure that a reasonable number of intestines with *Ascaris* and without *Ascaris* were obtained. A total number of 50 specimens and 19 specimens were examined, respectively.

Post mortem procedure. The small intestine was first stripped of worms and afterwards incised together with the stomach and the large intestine and rectum to obtain residual worms. The *Ascaris* were collected, separated into males and females, adults and juveniles detectable macroscopically and finally weighed. Samples of faeces for egg counts were taken from the rectum or if the latter was empty or the consistency of the contents was watery samples were taken from the large intestine in order to get samples which did not vary markedly in consistency. In any case the samples are referred to as faeces.

Faecal egg counts. Three g of faeces were weighed out and mixed thoroughly with 24 ml of a saturated NaCl-solution. The mixture was filtrated and a sufficient volume of the filtrate was withdrawn with a pipette and carefully run into one counting chamber (McMaster) After further stirring another sample was withdrawn and placed into the other chamber Thus a total volume of 0.3 ml containing 1/30 g of faeces was examined. Eggs were counted under the microscope, and the total number in the two chambers was multiplied by 30 to obtain the number of eggs per g of faeces. (*Henriksen* 1971)

Statistical evaluation. In order to examine the degree of correlation between the different parameters, the product-moment correlation coefficient (r) was computed according to *S kal & Rohlf* (1973)

RESULTS

It appears from Table 1 that *Ascaris* eggs were found even in pigs which did not harbour *A suum* at the time of slaughter

The findings in the 50 pigs harbouring *A suum* and which are summarized in Table 2 display that female worms are more frequent than males, and that the female *Ascaris* weigh more than the males which is in accordance with the greater size of the former

TABLE 1 *Faecal* A. Suum *Egg Counts in Pig With* *ut Intestinal Ascarians*

No. of pigs	Faecal egg counts (p.g)
1	<30
2	90
3	90
4	<30
5	1020
6	<30
7	<30
8	<30
9	30
10	30
11	<30
12	30
13	30
14	30
15	<30
16	<30
17	<30
18	<30
19	30

Key e.p.g Eggs per g of faeces

The correlation between the faecal egg counts and (1) the total number of *Ascaris*, (2) the total mass of *Ascaris* and (3) the ratio between male and female *Ascaris* is outlined in Figure 1 A, B, and C. It is evident not only from the correlation coefficient (r) but also from the mere plotting of the observations that there is a poor degree of correlation between the faecal egg counts and the three parameters mentioned above. By scrutinizing Figure 1 A and B it is found that correlation is best between the very low egg counts (<30 e.p.g) and the very low worm burdens, which is supported by the figures in Table 3 which shows the trend that egg counts below 30 e.p.g are generally associated with absence of worms or just a couple of *Ascaris*

Practically all eggs detected and counted were fertile regardless of whether they originated from pigs harbouring few or many eggs or adult worms.

Only 4 out of the 50 *Ascaris* infected pigs harboured evidently juveniles measuring from approximately 4 to 12 cm.

DISCUSSION

It has been customary for years in parasitological work, in practice as well as in experimental work, to carry out faecal egg counts in order to estimate the burden of parasites harboured by certain animals. It has been taken for granted that high egg counts reflected large worm burdens and *vice versa.* Egg counts may be influenced by the consistency of the faeces, diet, age of the hosts, sex ratio of the parasites, immunity administration of anthelmintics and other drugs applicated orally The recognition of these and other modifying factors together with the results from an increasing number of publications testifying the unreliability of egg counts as criteria of the size of worm burdens have largely been ignored in practice though it has been recommended not to let faecal egg counts form the basis of control programmes (*Honer* 1970)

TABLE 2. *Survey of* A. Suum *Burdens and Faecal Egg Counts (e p g) in Fifty Ascaris Infected Pigs at Slaughter*

No. of pigs	♂ no.	♂ w	♀ no.	♀ w	Total no.	Total w	Egg counts
1	0	0	12	15	12	15	2490
2	0	0	1	5	1	5	2370
3	1	2	5	20	6	22	3450
4	0	0	2	6	2	6	2190
5	1	2	12	51	13	53	3890
6	1	1	1	8	2	9	30
7	3	4	8	28	11	32	2160
8	5	6	10	40	15	46	360
9	6	11	1	4	7	15	240
10	3	5	2	13	5	18	150
11	5	6	7	24	12	30	840
12	3	6	5	30	8	36	240
13	1	2	4	10	5	12	1200
14	2	3	16	70	18	73	720
15	0	0	12	44	12	44	3900
16	2	4	5	25	7	29	60
17	0	0	1	5	1	5	30
18	5	9	5	21	10	30	450
19	2	4	3	21	5	25	90
20	1	2	9	65	10	67	4260
21	13	15	13	50	26	65	780
22	1	2	10	69	11	71	240
23	1	2	1	5	2	7	30
24	0	0	1	5	1	5	30
25	1	2	10	60	11	62	2220
26	10	11	13	42	23	53	1350
27	0	0	1	4	1	4	30
28	0	0	1	1	1	1	30
29	1	2	1	5	2	7	30
30	0	0	1	2	1	2	30
31	0	0	1	3	1	3	30
32	1	1	2	4	3	5	30
33	1(1)	2	4	20	5	22	2520
34	0	0	8(2)	29	8	29	30
35	6	14	4	26	10	40	660
36	3(1)	5	5(2)	20	8	25	1470
37	0	0	7	26	7	26	<30
38	18	27	16	62	34	89	1410
39	2	2	1	5	3	7	120
40	4	7	3	18	7	25	330
41	1	2	4	25	5	27	510
42	4	9	7	46	11	55	2850
43	4	4	8	20	12	24	2940
44	6	9	22(2)	69	28	78	10740
45	5	9	2	16	7	25	300
46		5	6	33	8	38	1170
47	3	7	4	25	7	32	1590
48	3	8	5	28	8	36	300
49	9	16	2	15	11	31	3540
50	0	0	5	27	5	27	1290

Key: no. number of worms w weight of worms in grams ♂ male worms ♀ female worms.
Numbers in brackets indicate juveniles.

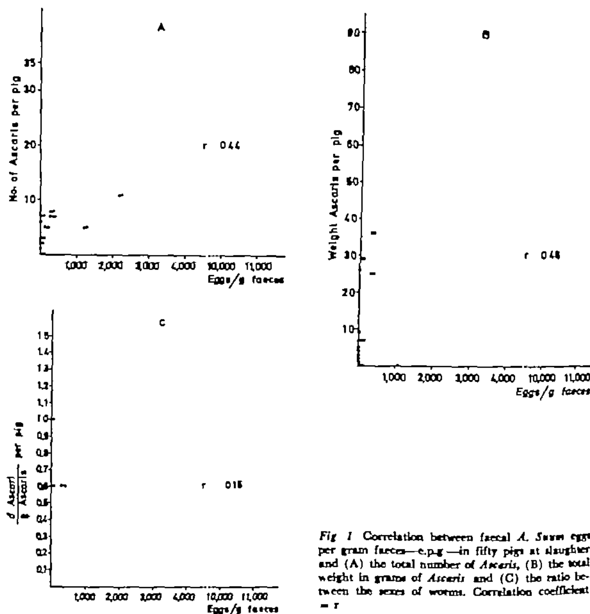


Fig. 1. Correlation between faecal *A. Suum* eggs per gram faeces—e.p.g.—in fifty pigs at slaughter and (A) the total number of *Ascaris*, (B) the total weight in grams of *Ascaris* and (C) the ratio between the sexes of worms. Correlation coefficient = r

It appears from a series of publications (*Michel* 1963, 1969 a-b-c, *Michel & Sinclair* 1969) dealing with the dynamics of *Ostertagia, i.e. that the egg output is fairly constant* regardless—within wide ranges—of the number of worms present. A theoretical and *mathematical approach to aspects of parasitism* including egg counts is published by *Whitlock* (1961), *Crofton* (1971 a b) and *Whitlock et al.* (1972).

The present investigation seems to display that faecal egg counts in porcine ascariasis *is of limited value as regards an evaluation* of the worm burdens. The obvious poor correlation between the egg counts and the para*meters mentioned* has the practical implication, *i.e.* that one cannot conclude from, say a high egg count, that the host harbours a considerable number of *Ascaris*. Consequently the statement that 1,000 *A. suum* e.p.g. are indicative of ascariasis (*Soulsby* 1965) by which it is obviously understood that the host in question is likely to suffer clinically in some way from the *A. suum* in-

TABLE 3 *Distribution of A. Suum in Pigs Classified According to Faecal Egg Counts*

Classes of egg counts (e.p.g.)	No. of pigs	Total no. of worms in individual pigs	Average
<50	22	0-0-0-0-2-0-1 2 1 1 1 1 1-0-3-0-0-0-0-0-8-7	1.3
50 – 1030	26	0-0-0-15-7 5-12-8-0-0-18-7 10-5-26-11 2-0-0-0-10-3-7 5-7-8	6.3
1030 – 2030	7	3-23-8-34-8-7 5	12.9
2030 – 3030	8	12 1 2 11 11 5-11 12	8.1
3030 – 4030	4	6-13-12 11	10.5
>4030	2	10-28	19.0

Key e.p.g. Eggs per g of faeces.

fection, may not be justified as 1,000 *A. suum* e.p.g may represent at any rate from 5 to 35 *Ascaris* (Figure 1 A) the former being of no clinical importance. However a very small number of eggs (<50 e.p.g) may indicate the presence of very few *A suum* (Figure 1 A)

The fact that eggs in some cases were detected in pigs without *A suum* implies that the pigs in question must have expelled their *Ascaris* burdens a short time before the examination for some reason or another An anthelmintic treatment is not likely to be responsible as it is hard to believe that farmers would treat their pigs a few days before slaughter Cases where fertile eggs were present in the faeces concomitant to the finding of only female worms in the gut raise the question for how long time female *A. suum* will be capable of shedding fertile eggs after the males have been expelled

Clapham (1936) *Ronéus* (1971) and *Andersen et al* (1973) have demonstrated that the smaller dose of *A suum* eggs, the higher percentage of the eggs will result in adult *Ascaris* in the intestine. The fact that *Jacobs* (1967) found that sows generally carry low *A. suum* burdens (on an average 3 worms per animal) made *Andersen et al* (1973) conclude that sows are particularly liable to give rise to patent infections in their offspring, and consequently they advocate a complete elimination of *A suum* burdens wherever practicable, but we do not know (1) whether "few eggs—many worms" is valid under natural conditions, as we are ignorant of the size of infective doses which give rise to natural *A suum* infections in piglets, and (2) whether the sows are the direct main source of the infection of their offspring Still more important in that respect, perhaps, is the problem of replacement of *Ascaris* burdens. In the present report, macroscopically detectable juveniles were only observed in 4 out of 50 infected pigs, which seems to permit the conclusion that adult *A suum* may not be continuously acquired to any considerable degree, otherwise the large majority of the pigs should be expected to have harboured intestinal stages of *A suum* varying very much in size and stage, as the animals are likely to have picked up infective *A suum* eggs daily throughout several weeks. The present findings suggest that once the *A suum* burden *is established* in a pig, the degree of contamination of the environment with eggs is of no importance as regards the development of a second and further infection resulting in adult *A. suum*, but may still be of importance as regards the degree of damage due to the migrating larvae.

Work is in progress to devise techniques suitable for a detection of *Ascaris* stages in the host originating from different infecti-

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the same, using *N. meningitidis* strain M1 of group B as the main test microbe. An *Escherichia coli* K12 strain was, as before, used for comparison in some of the experiments (11).

Purine-nucleoside phosphorylase EC 2.4.2.1 (2) was determined spectrophotometrically according to *Hoffmeyer & Neuhard* (7) by coupling the reaction with xanthine oxidase (EC 1.2.3.2) (2) whereby any liberated hypoxanthine is converted to uric acid. The reaction mixture contained the following in 3 ml: 300 µmoles potassium phosphate buffer pH 7.1, 6 µmoles inosine, 0.02 units of xanthine oxidase and 50 µl crude cell extract. The reaction was carried out at room temperature and the increase in absorbancy at 293 nm was followed in a Hilger-Gilford spectrophotometer in 1 cm cells. An increase in molar absorbancy of 12.0×10^3 may be used to calculate the amount of product formed (7). The extracts in this experiment were prepared from cells in exponential growth as described before (11). The only difference was that 2 mM inosine was used for possible induction of the enzyme instead of adenosine (3). Purine nucleoside phosphorylase (PNPase) was also assayed with radioactive adenosine, guanosine and inosine as substrates. Reactions were carried out in a total volume of 50 µl containing: 5 µmoles K-phosphate buffer pH 7.2, one of the following ribonucleosides: ^{14}C-8-adenosine 0.005 µmoles (52 µCi/µmole), C-8-inosine 0.005 µmole (53 µCi/µmole), ^{14}C-U-guanosine 0.004 µmole (65 µCi/µmole) (the latter compound was adjusted to this specific activity with the nonradioactive compound prior to use) and 10 or 25 µl crude extract made from cells harvested from blood agar plates (9). The reaction was initiated by the addition of extract and carried out at 37 C in a shaking water bath. The experiments were run for 5 minutes (*E. coli*) and 30 minutes (*N. meningitidis*) and stopped by cooling in an ice-water bath. The protein was precipitated with 50 µl ice-cold 96 per cent ethanol followed by 5 µl 0.1 M potassium EDTA and removed by centrifugation. Appropriate controls containing Tris/HCl buffer instead of phosphate and blanks with deproteinized extracts were included. Separation of the purine base from the corresponding nucleoside was achived by thin layer chromatography in 1 M NH acetate as specified in Table 2. Any radioactive ribose-1-phosphate (R-1-P) formed (from ^{14}C-U-guanosine) follows the solvent front. Radioautography and counting of the radioactive spots were as before (9, 11). The PNPase activity was also measured with ^{14}C-purine bases and R-1-P as substrates as described in Table 2.

Purine nucleosidase EC 3.2.2.1 (2) was, if not otherwise stated, assayed by mixing in a total volume of 50 µl: 5 µmoles Na-acetate buffer pH 5.4, ^{14}C-nucleosides as specified under PNPase and 10 or 25 µl crude extract in 0.05 M Tris/HCl buffer pH 7.2. The extracts were made from cells harvested from blood agar plates (9). The reaction was run for 30 minutes or as indicated in the individual experiment. The rest of the procedure was as described for purine-nucleoside phosphorylase. Blanks with deproteinized extracts were run in all chromatograms. Control experiments with uniformly labelled ^{14}C-adenosine were performed. These experiments were done with the following buffers: Na-acetate pH 5.4, Tris/HCl pH 7.4 and K-phosphate pH 5.7 and 7.4. Before chromatography of the supernatants in NH_4-acetate the cellulose layer was scraped off the plastic sheet in 1 mm channels between the origins to facilitate separation of the radioactive ribose formed. D-ribose follows the solvent front. Controls with chromatography of the supernatants were performed by ascending technique on cellulose thin layers in n-butanol-ethanol-H_2O (52:33:15 by vol.) for 3 hours (17 cm). R-1-P (and R-5-P) remains at the origin while D-ribose, adenine and adenosine have the R_F 0.41, 0.47 and 0.35 respectively. Further control was achived by two-dimensional chromatography using the isobutyric acid-conc. NH_3-H_2O-0.1 M EDTA solvent in the first direction and the n-butanol-acetone-acetic acid-conc. NH_3-H_2O solvent in the second direction, both for about 17 cm (9, 11). D-ribose, R-1-P, adenine and adenosine have the R_F 0.61, 0.47, 0.88 and 0.75 respectively in the first solvent and R_F 0.57, 0.30, 0.67 and 0.65 respectively in the second solvent. Parallel chromatograms were run in each experiment. Radioautography and identification were performed as previously described (10). Ribose was visualized on the chromatograms after radioautography by spraying with AgNO saturated acetone followed by ethanolic 0.5 per cent *NaOH*. Ribose phosphate was visualized by spraying with the molybdenic acid reagent of *Hanes & Isherwood* (4).

Purine-nucleoside kinase activity was assayed by mixing in a total volume of 50 µl: 3 µmoles K-phosphate buffer pH 7.4, 0.5 µmole $MgCl_2$, 2.5 µmoles KCl, 0.25 µmole ATP, 0.05 µmole ^{14}C-adenosine, inosine or guanosine and 10 µl crude extract (17, 24). In one of the experiments, the extract was the same as that used in the PNPase experiment shown in Table 2 (11). The experiments were run for 30 and 60 minutes. Otherwise the procedure was as explained in Table 2.

Adenosine deaminase EC 3.5.4.4 (2) was assayed as described for adenine deaminase (11) except that the reaction mixture included 80 µmoles of Na_2SO to inhibit any purine-nucleoside phosphorylase present (14).

Chemicals. ^{14}C-8-adenine (60 mCi/mmole), ^{14}C-8-hypoxanthine (59 mCi/mmole), ^{14}C-8-guanine (36.1 mCi/mmole), ^{14}C-8-adenosine (52 mCi/mmole), ^{14}C-U-adenosine (533 mCi/mmole), ^{14}C-U-guanosine (518 mCi/mmole) and ^{14}C-8-inosine (53 mCi/mmole) were obtained from The Radio-

chemical Centre, Amersham, Bucks. U.K. ^{14}C-2 xanthine (48 mCi/mmole) was from CEA, Departement des Radioelements, 91-Gif-Sur Yvette, France. Other fine chemicals were obtained from Koch-Light Labs. Ltd. Bucks. U.K. or from Sigma Chemical Corporation. St. Louis, Mo. U.S.A. The purity of the radioactive substrates was controlled as before (11) The ^{14}C-nucleosides were found to contain 1 to 2 per cent of the corresponding base. Before exposing whole cells to the nucleosides they were purified by chromatography on Whatman No. 1 filter paper (50 × 40 cm) in the n-butanol-ethanol-H_2O or NH -acetate solvent (1 M pH 7.0) (11) and eluted by chromatography with 0.5 M NH_4OH. The eluate was evaporated to dryness in vaccum, resuspended in the original sample volume and shown to contain no impurities on rochromatography

RESULTS

Labelling of purine metabolites from ^{14}C-8 adenosine When whole cells of *N meningitidis* were exposed to radioactive adenosine, label appeared in its corresponding purine base and 5 ribonucleotides (Table 1) When guanosine and inosine were the substrates, only faint spots on the film corresponding to these nucleosides could be seen (not shown) indicating that they are not metabolized by *N meningitidis*.

Purine nucleoside phosphorylase and purine nucleosidase Activities corresponding to PNPase could not be demonstrated in extracts from *N meningitidis* if inosine was

TABLE 1 *Labelling of Purine Metabolites from ^{14}C-8-adenosine in Intact Cells f* N meningitidis

Time of incubation sec.	Incorporation calculated as picomoles adenosine		
	Adenine	Adenosine	AMP + ADP + ATP
30	–	–	65.4
90	–	–	71.0
210	2.2	8	67.0

Batches of one ml culture in exponential growth in Medium KC (12) with appr 1.5 × 10⁹ colony-forming units were exposed to 5.2 µg ^{14}C-8-adenosine (spec. act. 52 µCi/µmole) for the time indicated. The rest of the procedure as described before (9 11)

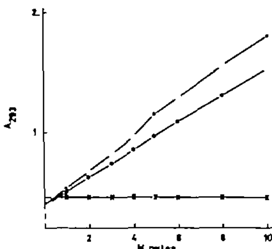


Fig 1 Search for purine-nucleoside phosphorylase in extracts from *N meningitidis* (×——×) Cells were grown with and without inosine. Each assay contained 0.37 mg protein. Control experiments with *E. coli* extracts. Cells grown with inosine (●——●) and without (○——○) Each assay contained 0.32 mg protein. Experimental conditions as described under Methods.

used as the substrate in the way it has been done in extracts from *E. coli* (Fig 1) Also, in contrast to the findings from *E coli*, no activity could be induced by inosine (see Methods) in *N meningitidis* (Fig 1) (5)

No significant PNPase activity could be demonstrated in extracts from *N meningitidis* if radioactive purine bases and R 1 P were used as substrates (Table 2) Using radioactive adenosine, guanosine or inosine as substrates, only adenosine was hydrolysed. This reaction had a higher activity in acetate buffer pH 5.4 than in K-phosphate or Tris/HCl buffer pH 7.2 (Table 3) The findings point to a specific adenosine nucleosidase in *N meningitidis* (2, 19) PNPase activity was easily demonstrated in extracts from *E. coli* by the same techniques (Tables 2 and 3)

Adenosine nucleosidase Table 4 shows the enzyme activity related to time. There is a gradual hydrolysis of adenosine yielding adenine and ribose. Fig 2 shows that the amount of adenosine hydrolysed is proportional to the amount of enzyme used.

TABLE 2. *Search for Purine-nucleoside Phosphorylase Activity in Dialysed Ext act from* N meningitidis *Using* ^{14}*C purine Bases and R 1-P as Substrates Control with Dialysed* E. coli *Extract*

Organism	Protein mg/assay	^{14}C-purine base added	^{14}C-purine base converted nanomoles
N meningitidis	0 07	Adenine	–
N meningitidis	0.07	Guanine	–
N meningitidis	0 07	Xanthine	–
N meningitidis	0.07	Hypoxanthine	–
E. coli	0 07	Adenine	54.3
E. coli	0.07	Guanine	7.2
E coli	0.07	Xanthine	9.9
E. coli	0 07	Hypoxanthine	53.6

Experimental conditions as described before (11) 50 µl reaction mixture contained: 3 µmoles Tris/HCl buffer pH 7.8 1.5 µmoles $MgCl_2$ 0 15 µmole R 1 P 0.05 µmole ^{14}C-purine base and 10 µl extract. The experiments were run for 30 minutes at 37 C with shaking in a water bath. Termination of the experiments, cellulose thin layer chromatography in 1 M NH -acetate pH 7 0 (17 cm) radioautography and counting of the radioactive spots were as before (9 11) R_F in the NH -acetate sol ent adenine 0.31 adenosine 0.51 guanine 0.34 guanosine 0.62, xanthine 0.39 xanthosine 0 65 hypoxanthine 0.55 and inosine 0 76 The bases had the following specific activities: ^{14}C-8-adenine 10 µCi/µmole, ^{14}C-8-guanine 6 µCi/µmole, ^{14}C-2 xanthine 5.5 µCi/µmole and ^{14}C-8-hypoxanthine 9.9 µCi/µmole, all adjusted to these specific activities with the nonradioactive compounds prior to use. The extracts used were made from exponentially growing cultures as described before (11)

* This is the sum of the adenosine, inosine and hypoxanthine formed (11)

TABLE 3 *Cleavage of* ^{14}*C purine Nucleosides by Crude Extracts from* N meningitidis *and* E. coli

Organism	Protein mg/assay	Time of incubation min	^{14}C-nucleoside in assay	Buffer and pH used in assay	^{14}C-nucleoside converted nanomoles
N *meningitidis*	0 17	30	Adenosine	Phosphate – 7.2	0.39
N *meningitidis*	0 17	30	Adenosine	Tris – 7.2	0.13
N *meningitidis*	0.17	30	Adenosine	Acetate – 5 4	1.36
N *meningitidis*	0 17	30	Guanosine	Phosphate – 7.2	–
N *meningitidis*	0 17	30	Guanosine	Tris – 7.2	–
N *meningitidis*	0.17	30	Guanosine	Acetate – 5 4	–
N *meningitidis*	0 17	30	Inosine	Phosphate – 7.2	–
N meningitidis	0 17	30	Inosine	Tris – 7.2	–
N *meningitidis*	0 17	30	Inosine	Acetate – 5.4	–
E coli	0 16	5	Adenosine	Phosphate – 7.2	1.27
E. coli	0.16*	5	Adenosine	Tris – 7.2	0.28
E. coli	0.16	5	Guanosine	Phosphate – 7.2	1.65
E coli	0.16*	5	Guanosine	Tris – 7.2	1.06
E. coli	0.16*	5	Inosine	Phosphate – 7.2	4.58
E coli	0 16	5	Inosine	Tris – 7.2	3.21

Experimental conditions as described under Methods.

* The *E coli extract had been kept frozen at –20 C for 3 weeks*

Maximum velocity of adenosine nucleosidase is obtained with adenosine at a concentration of about 1 mM under the experimental conditions used (Fig 3)

Adenosine nucleosidase activity in crude meningococcal extract in Tris/HCl buffer is very stable to heating (Fig 4) After exposure to 70 C for 5 minutes, about 72 per cent of the activity remained whereas no activity was left after treatment at 75 C.

TABLE 4 *Adenosin Nucleosidase Activity in Crude Extract from* N meningitidis

Time of incubation min	[14]C-adenosine not hydrolysed picomoles	[14]C-adenine formed picomoles	[14]C-ribose formed picomoles	The sum of [14]C-adenosine [14]C-adenine and [14]C-ribose picomoles
5	406	60	59	525
10	338	72	73	483
15	294	96	96	486
20	263	111	111	485
40	185	149	154	488
60	141	177	185	503

The *enzyme activity was assayed in acetate buffer pH 5 4 with* 0.5 nanomoles [14]C-U-*adenosine* (333 μCi/μmole) and 10 μl extract containing 0.05 mg protein. The extract had been kept frozen at −20° C for 20 days. Cellulose thin layer chromatography of the supernatants in 1 M NH acetate pH 7.0 (see Methods)

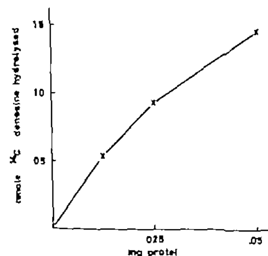


Fig 2 Activities corresponding to adenosine nucleosidase in meningococcal extract. Procedure as described under Methods except that the experiments were run for 60 minutes

Table 5 shows that the enzyme also is resistant to dialysis and freezing No significant change in activity was found after dialysis of the extract for 22 hours or freezing at −20 C for 2 months.

The adenosine nucleosidase was active over a pH range of 4 4–7 8 (Fig 5) the pH optimum being at 5 4 in acetate buffer The activity seems to some extent to be dependent on the type of buffer employed The activity in Tris/HCl buffer is lower than in phosphate buffer at the same pH (Table 3 Fig 5)

The effect of purine and pyrimidine nucleotides and end product inhibition were also examined (Table 6) Deoxyadenosine was the only nucleoside of those tested which had any effect on the enzyme activity In a concentration of 10 mM the activity was re

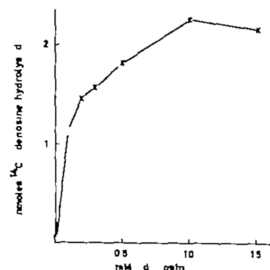


Fig 3 Adenosine nucleosidase activities in meningococcal extract. Effect of adenosine concentration. The specific activity of [14]C-8-adenosine was adjusted to 4.9 μCi/μmole with unlabelled adenosine. The rest of the procedure as described under Methods with 10 μl extract containing 0.075 protein.

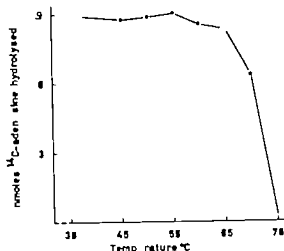


Fig 4 Activities of adenosine nucleosidase in extract from *N meningitidis* Portions of 0.6 ml extract prewarmed to 37 C, were placed in a water bath at the temperatures indicated for 5 minutes, chilled in an ice-water bath, and examined for activity Assay as described under Methods with 10 µl extract containing 0 05 mg protein.

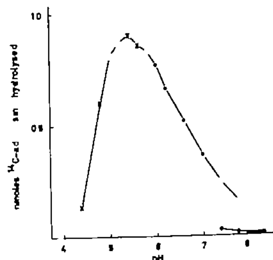


Fig 5 Effect of buffer and pH on the activity of adenosine nucleosidase in extract from *N meningitidis*. Na-acetate buffer (×——×) K-phosphate buffer (●——●) and Tris/HCl buffer (○——○) The assay as described under Methods with 10 µl extract containing 0.05 mg protein. The pH values are those given by the buffers at 37 C.

duced to 7.5 per cent. Among the end products, ribose showed little inhibitory effect at the concentration of 10 mM whereas the adenine inhibition was 95 and 100 per cent with 0.38 and 3 8 mM adenine respectively

There was no enhancement by phosphate (40 mM) or arsenate (10 mM) on the hydrolysis of adenosine (Table 7) The sulphhydryl reagents parachloromercuribenzoate (PCMB) and Cu^{++} both had inhibitory effects on the enzyme activity With PCMB (0 4 mM) the activity was 59 per cent and with Cu^{++} (1 and 10 mM) the activities were 74 and 7 per cent. The metal ion inactivating chemical fluoride (NaF) and ethylendiaminetetraacetate (EDTA) showed no significant inhibitory effects on the enzyme activity The divalent cations Mg^{++} Mn^{++} and Ca^{++} (all 10 mM) did not enhance the hydrolysis of adenosine (Table 7)

Purine nucleoside kinase Activities corresponding to kinases that phosphorylate adenosine, guanosine or inosine to their monophosphates were not detected in meningococcal extracts. Activities were searched for

TABLE 5 *Aden n e Nucleosidase Acti ity in Crude Ext act from* N meningitidis

Expt No.	Stat of extract	Protein mg/assay	^{14}C-8-adenosine hydrolysed nanomoles	Per cent activity
1	New	0.07	1.33	100
	Dialysed be n	0 07	1.30	98
2	New	0.05	0.89	100
	Kept frozen t 20° C for month	0.05	0.84	94

Experimental procedure as d bed under Methods.
* Extract dialysed against 100 lumes of 0.05 M Tris/HCl buffer pH 7.2 at 4 C.

TABLE 6. *Effect of Purine and Pyrimidine Nucleosides and Enzyme End Products on Adenosine Nucleosidase Activity in Crude Extract from* N meningitidis

Chemical added	Final conc. of chemical mM	^{14}C-8-adenosine hydrolysed picomoles	Per cent activity
None	–	893	100
Deoxyadenosine	10	67	7.5
Inosine	10	902	101
Guanosine	2	874	98
Xanthosine	2	849	95
Uridine	10	900	101
Cytidine	10	882	99
Adenine	0.38	45	5
Adenine	3.8	0	0
D-ribose	10	775	87

Experimental procedure as described under Methods. 10 µl extract contained 0.05 mg protein. The extract had been dialysed against 100 volumes of 0.05 M Tris/HCl buffer pH 7.2 for 22 hours at 4 C.

TABLE 7 *Effect of Phosphate Arsenate PCMB NaF EDTA and Divalent Cations on Adenosine Nucleosidase Activity in Crude Extract from* N meningitidis

Chemical added	Final conc. of chemical mM	^{14}C-8-adenosine hydrolysed picomoles	Per cent activity
None	–	895	100
Phosphate	40	900	101
Arsenate	10	828	93
PCMB	0.4	530	59
NaF	10	865	97
EDTA	10	843	94
$MgCl_2$	10	915	102
$MnCl_2$	10	891	99
$CaCl_2$	10	915	102
CuSO	1	659	74
$CuSO_4$	10	63	7

Experimental conditions as described under Methods. 10 µl extract contained 0.05 mg protein. The extract had been dialysed for 22 hours against 100 volumes of 0.05 M Tris/HCl buffer pH 7.2 at 4 C.

in several experiments in which radioactive adenosine was used as the substrate as shown in Table 8. In addition to the variations in experimental conditions shown in Table 8, the concentrations of adenosine, ATP and divalent cations were varied (1) but no activity was observed.

Adenosine deaminase The attempts to demonstrate this enzyme in *N meningitidis* were also altogether negative whereas control experiments with *E coli* were positive (Fig. 6) Using extracts from *N meningitidis* (not shown) there was no change in the absorption spectrum.

Control experiments with dialysed extracts from *N meningitidis* run with Tris/HCl buffer pH 7.2 and ^{14}C-8-adenosine for 30 minutes, chromatography of the supernatants and radioautography revealed only spots on the film corresponding to adenosine and adenine.

DISCUSSION

Whole cells of *N meningitidis* were found to metabolize exogenous radioactive adenosine (Table 1) while enzyme reactions for utilization of guanosine or inosine ...

TABLE 8. *Search for Adenosine Kinase in N meningitidis. Variations in Experimental Conditions*

Expt. series No.	Condition examined	Variations performed
1	Growth phase of the bacteria	Harvested from log phase and from stationary phase
2	Medium used	Growth in Medium KC and on blood agar plates (12)
3	State of extract	New dialysed and undialysed. Reduced glutathione or dithiothreitol added to the cells before or after sonication (17 ?8)
4	Buffer system used during the assay	Phosphate buffer pH 7 4 Tris/HCl buffer pH 7 4 Tris/maleate buffer pH 5.8 and acetate buffer pH 5.4 (8, 24)
5	Cations used during the assay	Mg^{++} or Mn^{++} (1 8) With and without K added (8, 17)

Fig 6 Change in ultra [illegible] absorption spectrum during incubation of *L* [illegible] with 150 μmoles Tris HCl buff [illegible] μmol [illegible] adenosine 80 μmoles Na SO [illegible] extract with 1 75 mg [illegible] (1.5 ml) was shaken [illegible] h for 90 minutes. The reaction [illegible] 1 5 ml 4 per cent HClO [illegible] ml of the supernatant was [illegible] recorded in a Hilger-G [illegible] from 240 to 280 nm (1) Adenosine added at the sta[illegible] sine added after deproteiniz[illegible] with HClO (●——●)

be absent. This is consistent with the fact that the purine mutant 6-1b (*his pro 4 G*) of *N meningitidis* could grow with adenosine instead of adenine, whereas no other purine nucleoside could substitute for its corresponding base (11) Also it must be concluded that enzyme reactions for utilization of xanthosine do not exist in *N meningitidis* since this nucleoside could not support growth, contrary to xanthine (11)

Purine nucleoside phosphorylases were first recognized by *Kalckar* (13) PNPases are widely distributed. They have been identified in several species of bacteria, yeast, mammals, chicken and fish (20) No PNPase activity was detected in meningococcal extracts. It appears from the data in Table 3 that, at a higher rate of adenosine hydrolysis in phosphate than in Tris buffer a phosphorolytic cleavage might seem reasonable. But no synthesis of adenosine from adenine and R 1 P could be found (Table 2) and exposure of extracts to uniformly labelled adenosine always showed radioactive spots on the film corresponding to adenine and ribose whereas the R 1 P (or R 5-P) spots remained unlabelled (see Methods) Both the synthesis and cleavage of the nucleosides with *E. coli* extracts showed the presence of the very active inducible purine nucleoside phos-

phorylase in this microbe (Tables 2 and 3 Fig. 1) (3 6, 11)

Since extracts from *N meningitidis* showed no enzyme activity towards the hydrolysis of guanosine or inosine (or xanthosine) the microbe seems to lack the enzyme purine nucleosidase EC 3.2.2 1 (2) which is found in yeast (5) lactobacilli (15 25) *Bacillus cereus* var *terminalis* (16) and in fish muscle (26) The enzyme catalyzing the hydrolytic cleavage of adenosine then must be adenosine nucleosidase EC 3.2.2.7 (2) which is reported to be specific for this nucleoside. It has previously been found in plant material such as wheat leaf (23) cabbage leaf (18) and Brussels sprouts (19)

Low pH optimum of adenosine nucleosidase (Fig. 5) is also found in plant material (23) The optimum in Brussels sprouts was in the region between pH 3.5 and 4.5 (19) As in *N meningitidis* the activity of the enzyme in sprouts was higher in acetate buffer than in other buffers (19) Both enzymes were stable during storage, freezing and thawing (Table 5) (19)

The hydrolysis of adenosine by extracts from *N meningitidis* was not affected by the presence of other nucleosides (Table 6) Similar findings have been reported to apply to the enzyme from Brussels sprouts (19) Deoxyadenosine seems to act as substrate for the meningococcal enzyme (Table 6) although this has not been tested directly Adenine was inhibitory to the Brussels sprouts and the present enzyme (Table 6) (19) The addition of F arsenate, Mg^{++} and Ca^{++} had no effect on either enzyme (Table 7) (19) Mn^{++} showed no effect on the meningococcal enzyme, but inhibited the enzyme activity from sprouts by 33 per cent in the same concentration. Cu^{++} and PCMB showed no effect on the sprouts enzyme whereas both were inhibitory to the meningococcal (Table 7) (19)

Adenosine nucleosidase in meningococcal extracts could not allow the synthesis of adenosine by ^{14}C-adenine and D-ribose. This is consistent with findings in other systems (5 25)

All attempts to demonstrate the presence of a nucleoside kinase in extracts from *N meningitidis* were negative. Adenosine kinase is found in yeast and mammalian systems (1) The enzyme is reported to be unstable, but was strongly stabilized during purification by dithiothreitol (17) The inability to demonstrate an adenosine kinase in *N meningitidis* extracts is not due to rapid dephosphorylation of AMP Incubation of meningococcal extract with ^{14}C-8-AMP and MgCl for 60 minutes in Tris/HCl buffer pH 7 4 chromatography of the supernatant, radioautography and counting showed the adenosine spot to be unlabelled and the adenine spot contained only about 2 per cent of the radioactivity originally added to the reaction mixture.

Inosine and guanosine kinases are found in micro-organisms such as *Salmonella typhimurium E. coli* and *Streptococcus faecalis* and in mammalian systems (1) The absence of these enzymes in *N meningitidis* reflects the inability of the purine mutant 6-1b to utilize inosine and guanosine for growth (11)

Adenosine deaminase is found in bacteria (7 21 22) and it occurs in tissues of both vertebrates and invertebrates (27) The deaminase is very active in *E. coli* extracts (Fig 6) (22) while *N meningitidis* obviously lacks this enzyme.

Thus, adenosine seems to be the only purine nucleoside that is metabolized by *N meningitidis* and it is hydrolysed by adenosine nucleosidase to adenine and ribose. Adenine can then serve as substrate for the adenine phosphoribosyltransferase present in *N meningitidis* (11)

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MYCOPLASMOSIS
EXPERIMENTAL PYELONEPHRITIS IN RATS

Demonstration of Antibody in Urine and Serum

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After inoculation of *M. arthritidis* strain P10-158 into the kidneys of 16 rats, inflammatory lesions developed in the papilla and cortex of 4 rats. In these 4 rats, antibodies were demonstrable in both serum and urine. In 6 rats, lesions were produced in the papilla only. With one exception, antibodies were found in the urine, but never in the serum of the rats in this group. In the remaining 6 rats, no lesions were produced, neither were antibodies demonstrated in serum or urine.

It has been shown that mycoplasmas may be cultivated from the upper urinary tract of patients suffering from chronic pyelonephritis (7, 8). Whether mycoplasmas may induce pyelonephritis under natural conditions is very difficult to prove. However experimental pyelonephritis can be produced in rats, using a strain of *M. arthritidis* (5). Experiments with this model have also given evidence of a synergistic effect between *M. arthritidis* and *E. coli* during the development of pyelonephritis (6).

A study of the immunological response to experimental pyelonephritis may provide useful information for a possible serodiagnosis of mycoplasma infections in the upper urinary tract of man.

The purpose of the present study was to examine the antibody response in serum and urine of rats experimentally infected with *M. arthritidis*.

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MATERIALS AND METHODS

Rats. Sixteen rats were used in the experiments. They were all male-rats (weight ~300 g) of the Sprague-Dawley breed and declared to be specific pathogen free. The rats were housed in single cages during the experiments.

Inoculation. A suspension in PBS pH 7.4 of *M. arthritidis* strain P10-158 was used. The suspension contained 10 colony forming units per ml (c.f.u./ml). 0.2 ml was injected directly into the left kidney according to the technique of *Møller & Robinson* (4).

Sampling of urine and blood. Every second day post inoculation (p.i.) the rats were transferred to disinfected cages. Urine was collected from the bottom of the cages within half an hour after voiding. After urine collection a blood sample was drawn from a tail vein. Part of each urine sample together with serum samples were stored at −70° C for subsequent serological examination.

Group A (rat 1–4) Inflammatory lesions In both papilla and cortex								
Rats no.	1		2		3		4	
Days post inoculation	Urine	Serum	Urine	Serum	Urine	Serum	Urine	Serum
2	0	0	0	0	0	8	0	NT
4	0	0	0	0	0	8	0	0
6	128	0	32	0	0	8	32	0
8	128	16	64	16	512	2	32	0
10	256	32	64	32	512	32	32	32
12	256	32	64	32	256	32	NT	NT
14	1024	16	64	32	256	32	NT	NT

NT Not tested.

Examination of urine Fresh urine was examined quantitatively for mycoplasmas by the plate agar counting method. The urine was also examined microscopically using 400 times magnification, for leucocytes.

Autopsy studies. Fourteen days p.i. the rats were euthanized. The left kidney was removed aseptically and 0.5 g tissue homogenized in 4.5 ml of PBS pH 7.4 The concentration of mycoplasmas in the tissue was determined by streaking 10-fold serial dilutions of the homogenate onto plates. Furthermore, 0.1 ml was deposited in semisolid medium from which plating was performed after 3 and 6 days of incubation. Blood agar plates were used for bacteriological examination. Incubation was performed at 37 C.

The remaining tissue of the left kidney was fixed in 4 per cent buffered formaldehyde and later on sectioned and stained with haematoxylin-eosin for histological examination.

Serological investigations. Urine- and serum samples were examined for antibodies against *M. arthritidis* (strain P10-158) by the indirect haemagglutination method using fresh erythrocytes (2) The supernate from a centrifuged sonicated suspension of the mycoplasma cells constituted the antigen. The urine- and serum samples were inactivated at 56 C for 30 min and absorbed with equal parts of packed washed sheep erythrocytes. All titrations were performed by means of a microtitre system (Cooke Engineering Co., Alexandria, Va.)

RESULTS

Group A In 4 rats (Group A rats Nos. 1–4) focal inflammatory lesions were found in the papilla together with more or less pronounced cellular infiltrations in the cortex, especially in the polar areas. The lesions were characterized by infiltrations mainly consisting of heterophilic granulocytes but also of some mononuclear cells.

In these rats, antibodies were found in both urine and serum (Table 1) Antibodies appeared in the urine 6–8 days p.i and in serum 2–4 days later With one exception (rat No. 4) the antibody titres were generally higher in urine than in serum.

Throughout the experiment mycoplasmas could be recovered from the urine as a rule in a titre of 10^3–10 c.f.u./ml. The concentration of mycoplasmas in kidney tissue was at the level of 10 –10^3 c.f.u./g Usually only a few leucocytes (1–5 per field of vision) were demonstrated in the urine at days 2 and 4 p.i. by microscopy In one rat (No. 3) however a very large number of leucocytes (20–100 per field of vision) was also found at days 8 and 10 p.i. From this rat, *Proteus* bacteria were cultivated from the kidney whereas no bacteria were found in the kidneys of the remaining rats.

Group B. In 6 rats (Group B rats Nos. 5–10) inflammatory lesions were confined to the papilla. The lesions consisted of foci of cellular infiltration similar to the findings in the group A rats.

In the group B rats, antibodies were found in the urine at days 6–8 p.i. (Table 1) except for one rat (No. 10) in which no antibodies were demonstrated. No antibodies

Inoculated with M. arthritidis *Strain P10-158*

Group B (rat 5–10) Inflammatory lesions in papilla							Group C (rat 11–16) No inflammatory lesions	
5	6	7	8	9	10	5–10	11–16	
Urine	Urine	Urine	Urine	Urine	Urine	Serum	Urine	Serum
0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0
64	0	0	32	32	0	0	0	0
256	128	32	32	32	0	0	0	0
256	128	64	64	32	0	0	0	0
256	256	64	64	32	0	0	0	0
256	128	64	32	16	0	0	0	0

were found in serum of any rat in this group. Mycoplasmas were intermittently cultivable from the urine during the experiment, mostly in a titre of 10^2–10^4 c.f.u./ml. In the kidney tissue, mycoplasmas were found in concentrations of 10^1–10^7 c.f.u./g. About one leucocyte per field of vision was found at days 2 and 4 p.i. in the urine of two rats (Nos. 5 and 8), whereas no leucocytes at all were observed in the urine of the remaining rats. Bacteria were not found in the kidneys of any rat in this group.

Group C. In 6 rats (Group C: rats Nos. 11–16) no histological lesions were found in the kidneys, neither were antibodies demonstrated at any time in urine or serum (Table 1). Nevertheless, mycoplasmas were recovered intermittently from the urine, usually in a titre of 10^2–10^4 c.f.u./ml. In two rats, no mycoplasmas were cultivable from the kidneys, while in the remaining 4 rats, mycoplasmas were recovered in concentrations of 10^2–10^4 c.f.u./g tissue. No leucocytes were demonstrated in the urine of these rats. Bacteria were not cultivated from the kidneys.

The results of pathological, serological and cultural examinations of all inoculated rats are summarized in Table 2.

TABLE 2. *Pathological, Serological and Cultural Findings in Urine, Serum and Kidneys of 16 Rats Inoculated with* M. arthritidis *Strain P10-158*

Groups of rats	No. of rats	Lesions (localization)	Antibody findings	Mycoplasma cultivated from urine	Leucocytes in urine No. of positive/total	Mycoplasmas cultivated from the kidneys No. of positive/total
Group A	4	Papilla and cortex	Urine and serum	Constantly	4/4	4/4
Group B	6	Papilla	Urine	Intermittently	2/6	6/6
Group C	6	0	0	Intermittently	0/6	4/6

Except one.

DISCUSSION

After inoculation of *M. arthritidis* into the kidneys of 16 rats, inflammatory lesions developed in 10 cases. In 4 rats (Group A) the lesions were present in the cortex as well as in the papilla of the inoculated kidneys. In this group of rats, antibodies were detected in both urine and serum. In 6 rats (G

B) the pathological changes were confined to the papilla. Five rats in this group developed antibodies, but in the urine only In one rat, antibodies were not measurable either in urine or in serum.

In 6 rats (Group C) no lesions developed in the inoculated kidneys, and antibodies were not demonstrated in any case.

Constant mycoplasmuria seems strongly related to the presence of pyelonephritic lesions in the kidney Intermittent mycoplasmuria, however may be found both in rats with lesions and in rats without lesions in the kidneys.

Demonstration of leucocytes in the urine seems to reflect inflammation in the kidney inasmuch as leucocytes were found in all of the rats with lesions in both papilla and cortex and also in two of the rats with lesions in the papilla only It is remarkable that the leucocytes appear early after inoculation (2 days p.i.) and that they disappear at the time when antibodies become detectable in the urine. In one case a high concentration of leucocytes occurred also when antibodies in urine were present (at days 8 and 10 p.i.) The demonstration of a concomitant bacterial infection in the kidney of this rat may however explain this finding

In mastitis experimentally produced by *M bovigenitalium* (1) antibodies were shown to appear in milk at the same time as the mastitis became clinically evident. In addition, the pathological changes in mastitis were characterized by an eosinophilic cellular response which was supposed to reflect antibody antigen reactions. Thus the antibodies formed during mycoplasma mastitis seemed to be of pathogenetic importance

In the experimental infection described in this study however no eosinophilia was observed and the leucocytes disappeared from urine when antibodies developed Therefore in contrast to the mycoplasma mastitis the antibodies developing during mycoplasma pyelonephritis are most likely without any pathogenetic significance although they may influence the course of infection

In view of the fact that antibodies were frequently found in the urine, without demonstrable antibody in serum, it seems reasonable to regard these antibodies as locally produced in the kidneys. This observation is in agreement also with the demonstration of locally produced precipitating antibodies in experimental *E. coli pyelonephritis* (3)

On the basis of the studies it may be concluded that inflammatory changes in papilla and cortex of kidneys experimentally infected with mycoplasmas are followed by an antibody production demonstrable in urine and serum. It may also be concluded that lesions in the papilla may be followed by antibody appearing in urine only and furthermore, if no lesions are induced, measurable antibodies are not produced despite the presence of cultivable mycoplasmas in the kidney

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A COMPARISON OF ANTIGENIC STRUCTURE AND PHAGE PATTERN WITH BIOCHEMICAL PROPERTIES OF *STAPHYLOCOCCUS AUREUS* STRAINS ISOLATED FROM HORSES

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Out of 70 *S. aureus* strains isolated from the anterior nares of horses, 48 (69 per cent) belonged to the E biotype. Approximately one third of these isolates were typed with factor sera, the 6 (35 per cent) that were typable showing 5 different patterns. All strains but one were non-typable with the basic sets of phages for typing human and bovine staphylococci even at RTD × 100. Without any exception the equine staphylococci of the E biotype contained polysaccharide Aα. Sixteen biochemically different strains belonged to the biotype A, B or C. A number of different serological patterns and phage patterns were found in these strains. In contrast to the typical horse-adapted staphylococci of the E biotype they had polysaccharide Aβ. The remaining 6 cultures could not be classified.

Biological characterization of equine staphylococci, as well as suggestions for their classification have been published by many authors (2, 6, 11, 17, 18, 21). Nevertheless, further studies are needed to provide a more extensive knowledge of ecologically different *Staphylococcus aureus* populations.

In a recent paper *Hájek et al.* (6) have described the physiological properties of staphylococcal strains isolated from the nares of horses. Most of the isolates were identified as belonging to the E biotype, which also includes staphylococci recovered from mink (7) and dogs (4). In the present study the biochemical properties of the equine strains are compared with their antigenic structure and sensitivity to phages.

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MATERIALS AND METHODS

From the nares of 43 horses admitted to the Olomouc Veterinary Hospital and of 122 healthy ones bred on five farms in Northern and Southern Moravia 70 *S. aureus* strains were obtained.

The subdivision of deoxyribonuclease-positive and coagulase-positive staphylococci was carried out according to *Hájek & Maršálek* (5, 10). Identification of biotypes was performed

TABLE 1 *Biochemical Properties of 70 S aureus Strains Isolat d from Horses*

Biotype		E	A	B	C	–
Number of strains		48	5	1	10	6
Deoxyribonuclease		48	5	1	10	6
Coagulation of plasma	rabbit	48	5	1	10	6
	human	0	5	1	10	0
	bovine	48	0	0	10	1
Fibrinolysin		0	5	0	0	0
Pigment		0	5	1	10	0
Haemolysin	α	0		0	1	1
	β	48	4	1	10	6
Crystal violet test	positive A	0	1	1	6	0
	negative C	0	4	0	4	1
	positive E	48	0	0	0	5

– Unclassified

of the following key criteria: the coagulase reaction in human and bovine plasma, the production of fibrinolysin, pigment, α- and β-haemolysins, and the growth type on crystal violet agar Detailed biochemical characteristics of the equine isolates are given in (6)

Slide agglutinations were performed according to the method of *Oeding* (8 13) All antisera were prepared by immunization of rabbits with human *S aureus* strains. Thirty-four selected strains were typed using factor sera a_1, a_2, b c e h h_2, l l_2, k k k_2, m 263-1 and 263-2 The presence of polysaccharides $A\alpha$ $A\beta$ $B\alpha$, $B\beta$, and C was examined by double diffusion in agar gel according to *Oeding* (14)

Phage typing was carried out with the basic sets of phages for typing human (19) and bovine (20) staphylococci. Strains resistant to RTD were retested in RTD × 100.

RESULTS

Forty-eight (69 per cent) of the 70 staphylococci that were deoxyribonuclease-positive and coagulase-positive in rabbit plasma belonged to the E biotype. All the biotype E strains coagulated bovine but not human plasma, lacked fibrinolysin, pigment, and α-haemolysin, produced β haemolysin and grew with positive (white) colonies on crystal violet agar (Table 1)

Seventeen isolates of the E biotype were typed serologically Out of these, 6 strains (35 per cent) agglutinated in factor sera showing 5 different patterns.

All 48 strains except one which was lysed by phage 42E at RTD × 100 were non typable by the phages here used (Table 2)

Without any exception the equine staphylococci of the E biotype contained polysaccharide $A\alpha$ only (Table 4)

Six non-pigmented staphylococcal strains could not be classified. They were resistant to the phages used Two strains contained the polysaccharide $A\alpha$. One of these most likely a deficit variant of the E biotype, differed only by a negative coagulase reaction in bovine plasma. The other formed α haemolysin

TABLE 2. *Incidence of Agglutinogens and Phage Patterns in 54 Non-Pigmented S. aureus Strains Isolat d from H ses*

Biotype	Number of strains	Serological pattern	Phage pattern	Phage group	Total
E	48	a	42E	III	1
		h_1	NT		2
		h_1/ l_3	NT		1
		263-1	NT		1
		a_3/ h_1/h_2/l l_2/m/n/263 1/263 2	NT		1
		NT	NT		11
		NE	NT		31
–	6	NT	NT		1
		NE	NT		5

NT Non-typable NE Not examined – Unclassified.

TABLE 3. *Incidence of Agglutinogens and Phage Patterns in 16 Pigmented S. aureus Strains Isolated from Horses*

Biotype	Number of strains	Serological pattern	Phage pattern	Phage group	Total
A	5	$a_y/c_y/h_y/l\ k$	3A/3C/55/116	II	1
		263-1	84/85	III	1
		a_y/h_y	NT		1
		$b\ /c_y/h_y/261\ 1$	NT		1
		c	NT		1
B	1	n	42E/83A/81	III	1
C	10	$a_y/c_y/m/263\ 1$	NT		1
		b_y/n	29/52/79/55/71/6/ 42E/47/54/81	I/II/III	1
		$c_y/\ /263\text{-}1$	85	III	1
		h_y/n	3C/42E/47/75/77/81	II/III	1
		$k\ k_y/m/n$	85	III	1
		m/n	83A	III	1
		n	29/79	I	1
		NT	79/55/42E/47/ 102/116	I/II/III	1
		NT	83A	III	1
		NT	42E/85	III	1

NT Non-typable

and grew with negative (violet) colonies on crystal violet agar The 4 remaining unclassified strains lacked coagulase activity in bovine plasma and contained polysaccharide B, one of them polysaccharide C in addition

Among the group of 16 pigmented strains 5 belonged to the A biotype, 1 to the B biotype and 10 to the C biotype (Table 1) Thirteen of these strains were agglutinated by factor sera and 12 were typable by phage. A number of different patterns were found (Table 3)

TABLE 4. *Incidence of Precipitinogens in 70 S aureus Strains Isolated from Horses*

Biotype	Number of strains	Polysaccharide				
		Aα	Aβ	Bα	Bβ	C
E	48	48	0	0	0	0
A	5	2	5	0	0	0
B	1	0	1	0	0	0
C	10	0	10	0	0	0
–	6	2	0	4	0	1

– Unclassified

In contrast to the 48 strains of the E biotype all the 16 pigmented strains had polysaccharide Aβ and two strains of the A biotype polysaccharide Aα in addition (Table 4)

DISCUSSION

The majority of the *S aureus* strains (69 per cent) isolated from the anterior nares of horses had congruent biochemical properties (6) According to the classification proposed by *Hájek & Maršálek* (5 10) these strains belonged to the E biotype. This biotype also includes staphylococci of mink (7) and of canine (4) origin.

Resistance both to human and to bovine phages seems to be a characteristic feature of equine staphylococci of the E biotype as well as of strains of the same biotype obtained from dogs (16) and mink (15) Some authors (2, 17) have found in their sets of staphylococci of equine origin rather significant numbers of typable strains, but these were almost always pigmented ones, probabl transferred to horses from other animr

man. These pigmented staphylococci which had not been subdivided in more detail, probably belonged to the biotypes A, B or C.

The *agglutination in factor sera* prepared against human *S. aureus* strains also revealed a low typability of the E biotype strains examined. Further the demonstration of 5 different patterns among the 6 typable strains indicated a high degree of independency of the strains. It was felt that the information which might be gained through the serological typing of all the E biotype strains would *not justify the effort involved.*

Similar poor results were obtained by serological typing of mink isolates (15) only 17 per cent of positive agglutinations, without any definite pattern, being exhibited.

Canine staphylococci belonging to the E biotype behaved, however quite differently in factor sera. Eighty four per cent of these strains were easily typable, possessing a_4 and k_1 *as the dominant antigens* (16)

Using absorbed serum prepared against a representative strain of the corresponding host origin *Yoshimura* (21) reported 60 per cent agglutinability of equine staphylococci. *Shimizu* (18) as well as *Ochi* & *Shimizu* (12) demonstrated a specific antigen (W) in more than 90 per cent of biochemically identical equine and canine cultures, this antigen not being demonstrated in isolates belonging to other biotypes.

Precipitation in agar gel exhibited results that coincided remarkably well with the classification performed by biochemical methods. All strains of the E biotype differed clearly from the others, containing polysaccharide Aα only

Other polysaccharides were demonstrated in E biotype strains of canine and mink origin (14) In these collections of strains, therefore, the equine ecotype (forma specialis) as well as the canine and mink ecotypes, were characterized within the scope of the E biotype through their wall polysaccharides.

In contrast to the equine strains of the E biotype the strains of the other biotypes contained polysaccharide *Aβ and two out of* five strains of the human A biotype contained polysaccharide Aα in addition. The *combination of these two precipitinogens* is rather regular in human staphylococci (14)

In 4 isolates unclassified by biochemical methods, polysaccharide Bα, the specific teichoic acid of *S. epidermidis* was demonstrated (1 3 9) A remarkably low biochemical activity (6) was observed in these cultures. Summarizing the biochemical and serological data, it seems that these 4 cultures belong to *an intermediary group* ranked between the species *S. aureus* and *S. epidermidis*

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INTERFERON PRODUCTION AND PREVENTION OF VIRAL INFECTIONS IN MICE BY COMPONENTS OF A MIXED BACTERIAL VACCINE

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The bacterial components of the mixed standard bacterial vaccine (SBV) were tested for protective effect against influenza A_2 and Vesicular stomatitis virus (VSV) infection in mice as measured by mortality and survival time. *Klebsiella aerogenes* and *Haemophilus influenzae* protected against both viruses, *Staphylococcus aureus* against VSV and *Neisseria* against influenza virus. Following injection of *Klebsiella aerogenes*, *Haemophilus influenzae* and *Staphylococcus aureus* significant titres of interferon (IF) could be detected in the serum. Correlation between protective effect and IF production was not complete, which may indicate that other host defence mechanism(s) are also activated by the vaccine components.

In an earlier communication we have reported, that a standard bacterial vaccine (SBV) produced circulating interferon (IF) when injected intraperitoneally (i.p.) into mice (5). It has also reduced the mortality caused by intranasal (i.n.) inoculation of influenza A (2) and Vesicular stomatitis virus (VSV) (5) or in some experiments extended the survival time after infection. Furthermore, vaccine treatment a few hours prior to viral inoculation reduced development of gross pulmonary lesion, [illegible] growth of virus in the lungs and antibody response against the virus. Our findings [illegible] firmed by other authors (17) using bact[illegible] [illegible]ines of composition similar to our [illegible]. These vaccine protected mice against infection with parainfluenza 1 Sendai virus and Semliki forest virus (11).

We have suggested (5) as did *Singer & Hardegree* (17) that the protection may at least in part be mediated by production of IF. Alternative explanations may be adjuvant effect and nonspecific stimulation of the humoral and cellular immunity. These mechanisms are currently investigated in our laboratory. The protective effect in both alternatives is assumed to be mediated by nonspecific stimulating mechanisms, while stimulation of the specific immune response against the various bacterial antigens seems [illegible] be of minor importance. Therefore it is [illegible] interest to select the active components of the vaccine which are protective against viral infections and which stimulate the host defence mechanisms in question. In the present communication we report the protective ef

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fect of the individual bacterial components of the mixed standard bacterial vaccine against infection by influenza A_2 virus and the VSV and the ability of these components to induce the *in vivo* production of IF in mice.

MATERIALS AND METHODS

Mice NMRI albino mice were originally obtained specific pathogen free (SPF) from the National Institute of Public Health, Oslo. For IF induction and for influenza virus infection, young adults were used and, for infection with VSV 2–3 week old mice of either sex were used. Four to six suckling mice were kept in one cage with one mother during the observation period.

Virus: Influenza A_2/Singapore 1/57 strain was given to us by Dr. *K. Herzberg*, Frankfurt a.M., West Germany In our laboratory the virus was passed once in the allantoic cavity of 10 days old embryonated eggs. The allantoic fluids were harvested 3 days after inoculation. Allantoic fluids with high haemagglutination titres were pooled and stored at –70 C. The infectious titre was tested *in vitro* in African green monkey (Vero) cells and *in vivo* in mice, by intranasal inoculation of 0.1 ml of the virus.

Vesicular stomatitis virus (VSV) Indiana strain was passed 2 times in the allantoic cavity of embryonated hens eggs in our laboratory The allantoic fluids were pooled and stored at –20° C. The infectious titre was estimated in L-F mouse fibroblast cells by the micro- end point titration method.

Bacterial vaccine The Standard Bacterial Vaccine (SBV) produced by the National Institute of Public Health, Oslo is composed of the following bacterial species *Staphylococcus aureus Streptococcus pyogenes Streptococcus viridans Streptococcus pneumoniae Haemophilus influenzae Klebsiella aerogenes* and *Neisseria* sp. Formalin killed suspensions of these components were kindly given to us by Dr *E. T* [illegible] Oslo. The components were used in a concentration roughly equivalent to the total number of bacteria in the standard vaccine (1.8 × 10⁹ bacteria per ml)

Interferon assay: Blood was obtained from the axillary vein at times indicated in the text. Blood from two or three mice was pooled for each sampling. Serum was separated after clotting Serum samples were kept at –20° C until tested. All tests were done simultaneously Prior to testing, serum samples were diluted 1 5 in Eagle Hanks minimal essential medium (MEM) adjusted to pH 1 which resulted in final pH of approx. 2 0. After 2 days at 4 C the samples were neutralized by 1N NaOH. Interferon activity in the sera was assayed by means of the infectivity inhibition test, employing the micromethod described in detail elsewhere (3) In brief twofold dilutions of the sera were added to freshly seeded L-F_1 cells in plastic microtrays. After incubation overnight, the test cups and the control cups without serum were challenged with approximately 10 mean tissue culture infectious doses ($TCID_{50}$) of VSV A back titration of the challenge virus was run in parallel. After sealing, the trays were incubated at 37 C. Microscopical reading was done after 3 days when the end point titration of VSV was complete. The IF titre was estimated as the highest dilution which inhibited viral cytopathogenic effect in 50 per cent of the cups, calculated by the method of Reed and Muench. The titres were calibrated to 10 $TCID_{50}$ of challenge virus by means of the standard slope of regression line for mouse IF tested in the VSV/L-F_1 system, as described in detail elsewhere (1) One unit according to this system equals approximately 2.8 units of the international standard received from the National Institutes of Health, Bethesda.

Statistical methods Differences in mortality were analysed by a χ^2-test. To evaluate the length of survival, Wilcoxon two sample test was used.

RESULTS

Effect of Vaccine Components on Mortality Caused by VSV

Baby mice were injected i.p. with 0.1 ml of a suspension containing one bacterial component. The groups included 10 to 17 mice, at least two litters housed separately each with one mother Four hours later all groups were inoculated i.n. with approximately 10 LD_{50} VSV without anaesthesia. One group was only given virus, but no vaccine. Mice dying within one day after inoculation were excluded from the experiments because deaths were considered to be due to manipulation during inoculation. All groups were observed for 14 days and mortality was recorded daily From the third day after inoculation the mice began to develop the characteristic picture of VSV infection and deaths began to occur (Fig. 1) Significantly fewer mice died in the groups injected with *Klebsiella aerogenes*, with *Haemophilus influenzae* and with *Staphylococcus aureus* The other components had no significant effect on mortality neither did they prolong the mean survival.

TABLE 3 *Protection Against Viral Infections and Maximal Interferon Titres Induced by the Standard Bacterial Vaccine and Its Components**

Agent	Protection against		Interferon titre
	Influenza A_2 (survival time)	VSV (mortality)	
Neisseria	<0 05**	<0.1>0.05	25
Str pyogenes	>0 1	>0 1	<20
H influenzae	<0.05	<0.01	90
Staph. aureus	>0.1	>0.05	140
Klebsiella	<0 05	<0.05	70
Str pneumonia	>0.1	>0 1	45
Str viridans	>0 1	>0.1	20
SBV	<0 05	–	75

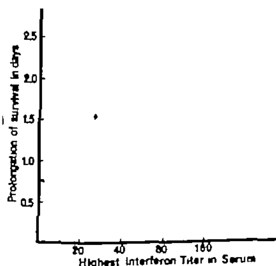
* Summary of the results of Fig. 1 and Tables 1 and 2

** Probability of no difference from controls.

from the IF production alone (Fig 2) while staphylococci were not protective in spite of relatively good IF production.

DISCUSSION

Four species had protective effect in the present experimental system. *H influenzae* and *K. aerogenes* against both viral infections, *S aureus* against VSV and *Neisseria* against influenza A_2 virus. The protection was to a certain degree correlated with production of IF but this correlation was far from complete. The endotoxin rich *K aerogenes* and *Neisseria* protected more, and *S aureus* protected less than otherwise to be expected from the IF production alone. It is possible that the various components stimulate production or release of quantitatively different IF Neither it is unlikely that different mechanisms of host defence are activated by the different bacterial agents. Stimulation of IF production is one of these mechanisms, adjuvant effect and nonspecific stimulation of cellular and/or humoral immune apparatus might be others. SBV as well as *K aerogenes* and *H influenzae* reduced the development of infection caused by both viruses and they also stimulated production of significant amounts of IF IF stimulation may be the mechanism by which the antiviral resistance was mediated in these cases, although it is doubtfull whether it is the full and only explanation of the effect. To obtain similar effect with exogenous IF much higher titres were required (unpublished observations)



F g 2 Correlation of the maximal interferon titres and protection against influenza A_2 irus infection after intraperitoneal injection of the individual components of SBV Protection is measured by the prolongation of survival time. Symbols as in Fig 1

Neisseria protected only against influenza A virus, but not against VSV which is more sensitive to the IF action. As these bacteria stimulated only production of marginal titres of IF it seems therefore unlikely that the protective effect is mediated by IF On the other side, staphylococci stimulated IF pro-

duction and protected against the IF sensitive VSV but not against the less sensitive influenza A virus. This might represent an IF mediated protection.

It has been demonstrated by several authors that administration of various bacteria, especially Gram-negative species could inhibit or attenuate viral infections (9) The same bacteria may also stimulate IF production in various experimental animals. Among the components of SBV these effects have been demonstrated earlier in *H influenzae* and various members of the genus Enterobacteriaceae (6 8, 12, 14 15 18) The active component of these bacteria is most likely the endotoxin complex (9) A glycolipid moiety of the lipopolysaccharide complex was recently reported to produce IF (19) Other parts of bacteria, proteins, lipids, carbohydrates and nucleic acids may also excert similar activities (4 10 13)

The effect of staphylococci is more difficult to explain. IF stimulation in experimental animals has not been described earlier Protection against vaccinia virus infection in mice by staphylococcal phage lysate has been demonstrated, without presence of IF in the serum (16) It should be mentioned that in these experiments, serum samples for IF tests were obtained 24 or 48 hours after injection. At the present time, the staphylococcal component(s) which stimulated IF production, remains obscure and we do not know whether additional mechanisms are activated. *In vitro* inhibition of Sindbis, Western equine encephalitis and West Nile viruses by a constituent of an unidentified staphylococcus has been described earlier (7) The inhibitor probably a polysaccharide, acted directly on the viral replication in the monolayer cultures, and it did not induce production of IF in the cell culture system. A direct action in our *in vivo* model cannot be excluded at the present stage of investigation, although SBV including staphylococci given 3 hours after influenza virus inoculation had no effect on the development of infection (2)

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BRIEF REPORTS

THE EFFECT OF *EPERYTHROZOON COCCOIDES* INFECTION ON THE NUMBER OF PLAQUE FORMING CELLS IN MICE

I. Ljungström, G. Huldt and A. Moller

Many laboratory stocks of mice are infected with the Bartonella-like parasite *Eperythrozoon coccoides*. The infection is usually overlooked as the only obvious sign is a moderate chronic splenomegaly. However 3 to 4 days following splenectomy the parasites appear in the blood and can be seen in Giemsa-stained smears as blue dot-like bodies clustered on the surface of the erythrocytes and sometimes encircling the cells. In heavy infections also free eperythrozoa are seen.

It has been known for a long time that concurrent *E. coccoides* infections alters the course of other induced infections. Rodent malarias may be reduced in virulence (6) as are some viral infections (10) whereas other viral infections may be made more severe (3, 8).

Recently studies by *Finerty et al.* (2) have suggested that concurrent *E. coccoides* infection reduces the humoral response to *Plasmodium berghei* and they suggest that antigenic competition is the possible reason.

In the light of the above-mentioned observations which suggest that *E. coccoides* infection can affect immune responses we decided to investigate the influence of the infection on the number of antibody producing cells detectable after immunization of the host with sheep erythrocytes. The antibody producing cells were measured by the plaque formation test of *Jerne et al.* (4).

Materials and Methods

Animals. Two lines of 8 weeks old CBA mice were used. These were designated CBA/LA and CBA/SBL. The lines were derived from the same parent line, but have been bred and maintained separately for about 15 and 10 years, respectively. Groups of mice from each line were splenectomized and blood films were made 1, 3 and 5 days later. These were stained by Giemsa and were examined for the presence of *E. coccoides* on the erythrocytes. Almost all the CBA/LA were found to be infected whereas all the CBA/SBL were free of infection.

In two experiments a comparison was made of the antibody response to SRBC in CBA/LA mice with chronic *E. coccoides* infection and CBA/SBL mice free of infection.

In a third experiment a comparison was made of the antibody response to SRBC in CBA/SBL mice experimentally infected 6 days earlier with *E. coccoides* and CBA/SBL controls free of infection.

Plaque assay. Groups of 12 mice were immunized intra-peritoneally with 0.2 ml of a 25 per cent suspension of washed sheep erythrocytes. Four days later half of each group were killed and the spleens removed aseptically and were weighed. The spleens were then teased and dilutions of 1×10^{7} and 5×10^{6} cells/ml balanced salt solution from the individual spleens were prepared. The plaque forming capacity of spleen cells from non-immunized control mice also was tested. In each experiment 1×10^{6} spleen cells from each of 4–7 such mice was included.

After 4 days, 6 mice in each experimental group were killed and the number of direct plaques, mainly of IgM class (5), was assayed according to *Jerne et al.* (4) with modifications described by *Pasanen & Mäkelä* (5). After 14 days, the remaining mice in each group were killed and spleens were treated as above. This time an indirect plaque assay was conducted by adding a rabbit anti-mouse gammaglobulin before incubation. This allowed plaque forming cells of the IgG class to be assayed (1, 9).

Results and Discussion

As can be seen from Table 1, all eperythrozoon infected mice had significantly larger spleens than uninfected controls. In experiments 1 and 2, more direct plaques were formed by spleen cells from infected mice than by spleen cells from the non-

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TABLE 1 *Spleen Index and Plaque Formation in Immunized Norm l and Infected Groups of CBA Mice*

	Mice	Experiment 1		Experiment 2		Experiment 3	
		CBA/L0 Chronic inf.	CBA/SBL Normal	CBA/L0 Chronic inf	CBA/SBL Normal	CBA/SBL Acute inf.	CBA/SBL Normal
	No. of animals	6	6	6	6	6	6
4 day	Spleen Index = $\frac{\text{spleen weight} \times 100}{\text{body weight}}$	0.89±0.04	0.44±0.05	0.68±0.02	0.41±0.02	1.08±0.03	0.62±0.03
		(p = .001)		(p = .001)		(p = .001)	
4 day	(Direct) Plaque forming cells Per 1×10^5 ± SE	88±5	24±8	127±10	54±7	109±12	41±11
		(p = .001)		(p = .001)		(p = .001)	
14 day	Spleen Index = $\frac{\text{spleen weight} \times 100}{\text{body weight}}$	0.37±0.02	0.36±0.03	0.50±0.05	0.44±0.05	0.96±0.03	0.31±0.01
		(p = .001)		(N.s.)		(p = .001)	
14 day	(Indirect) Plaque forming cells Per 5×10^6 ± SE	56±12	86±10	106±36	120±26	93±5	49±14
		(N.s.)		(N.s.)		(p = 0.01)	

TABLE 2 *Plaque Formation in Non-immunized Normal and Infected Groups of CBA Mic*

	CBA/L0 Chronic Infection	CBA/SBL Normal	CBA/SBL Acute Infection	CBA/SBL Normal
Direct plaque forming cells per 10^6 ± SE	28.0±4.3	2.4±0.5	29.3±5.6	2.4±0.5
	(p = 0.001)		(p = 0.001)	
Indirect plaque forming cells per 10^6 ± SE	0.25±0.16	1.3±0.4	1.5±0.5	1.3±0.4
	(p = 0.01)		(N.s.)	

infected controls. In experiment 3 the spleen cells from the infected mice showed higher plaque forming capacity both in the direct and indirect tests.

Table 2 shows plaque formation by spleen cells from non-immunized animals. In the direct test, significantly more plaques were developed by spleen cells from mice with acute and chronic eperythrozoon infection. However this will only make a negligible contribution to the differences in the immunized animals since this test on the non-immunized animals was performed with 1×10^{6} cells/ml while only 1×10^{5} cells/ml were used in the test on immunized mice.

These results indicate that an inapparent chronic infection of *E. coc oides* as well as acute infections in mice can drastically affect their antibody response to SRBC. There was a difference, however between the acute and chronic infections. The acute infection led to an enhancement of both IgG and IgM plaque forming cells to sheep erythrocytes whereas the chronically infected mice had only an enhanced IgM response.

We have no evidence to suggest the mechanism for the enhancement of the antibody response but the splenomegaly may be related to it. In some human situations, gross splenomegaly is associated with elev ted IgM responses (7). On the other hand, it cannot be excluded that the demonstrated increase in anti-SRBC, antibodies is due to cross reactivity between SRBC and parasite antigen. Further studies on the responsiveness of eperythrozoon infected mice to unrelated antigens are in progress.

The main importance of the present work is that it serves as an example of an altered response to an antigen where the responsible agent could easily be overlooked. It is possible that differences between some strains of mice in their immune responses may be related to the fact that some strains are infected with organisms such as *E. coccoides* whereas others are free from the infection. This study should emphasize the necessity both for ex amination for pathogens and for good animal-house hygiene in all animal experimental work. This is true particularly when the animals are used for immunological experiments.

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PROPERDIN ACTING AS A C3 CONVERTASE

Ulf Johnsen

Properdin is a basic euglobulin appearing in normal human serum in an inactive state. The concentration has been calculated to be about 25 µl/ml (*Götze & Müller-Eberhard* 1974). Conversion of inactive properdin (P) to its active form ($\overline{P}$) is mediated by several naturally occurring polysaccharides and by lipopolysaccharides.

Properdin acts in the early stages of the alternate pathway of complement activation. The exact reaction mechanism has not yet been fully clarified.

Materials and Methods

Purification of properdin. Properdin was purified in its active form in the following way. Euglobulin was prepared from fresh human serum by precipitation in an acetate-buffer, ionic strength 0.02, pH 5.4. Active precipitate was dissolved and passed through a DEAE-column. Active fractions were concentrated and put on a Sephadex G-200 column. Active fractions appeared in the slope of the 19S peak.

Testing for properdin activity was performed as described by *Götze & Müller-Eberhard* (1974). C3 conversion indicates presence of activated properdin.

The purified product reacted with specific antisera to properdin. The preparation did not give any precipitation line when tested with specific antisera to C3, C3-proactivator (C3PA), C1s and plasminogen, respectively. With anti-whole human serum a weak precipitate appeared representing small amounts of IgG in the preparation. No IgA or IgM were detected.

Purification of C3. C3 was purified essentially according to the method of *Nilsson & Müller-Eberhard* (1965). The purified C3 gave only one precipitation line against anti-whole human serum. The preparation did not contain any detectable amounts of C3b.

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Preparation of C3b. C3b was prepared by short time trypsination of the purified C3 according to *Cochrane & Müller-Eberhard* (1968).

Detection of C3-conversion products. Crossed immunoelectrophoresis according to *Laurell* (1965) and *Ganrot* (1972) was used to detect conversion of C3. C3c (β_{1A}) was distinguished from C3b by its higher electrophoretic mobility.

Antisera

Antisera against C3, C3PA and C1s were prepared according to *Sjöholm* (1974).

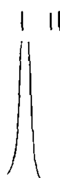
Antiserum to plasminogen was kindly supplied by *C. B. Laurell*, Malmö General Hospital, Malmö, Sweden.

Anti-whole human serum was purchased from Behringwerke AG, Germany. *Antiserum to properdin* was raised in rabbits by repeated subcutaneous injections with the properdin preparation. The final anti-properdin was contaminated with small amounts of anti-IgG. The specificity of the antiserum was kindly tested by Dr. *P. J. Lachmann*, London.

Results

Purified C3 and purified properdin were mixed and incubated at 37° C for 30 minutes. Purified C3 was incubated in parallel with this. Analysis of the mixture by crossed immunoelectrophoresis showed a conversion of C3 to a fragment with the same electrophoretic mobility as C3b. As reference C3b, obtained from trypsinated C3, was used. Analysis of the incubated C3 preparation did not reveal any conversion. The same results were obtained with two different preparations of C3 and properdin (Fig. 1). The C3 conversion mediated by active properdin was independent of Mg^{++} and Ca^{++} and was not inhibited by the addition of soya bean trypsin inhibitor (SBTI). The conversion of C3 in the presence of activated properdin was found to be proportional to the amount of properdin used.

Precipitation with IgG-anti-properdin eliminated or diminished the C3 converting ability of the properdin preparations.



A



B



C



D

1 23

Fig 1 Crossed immunoelectrophoresis with anti-C3 present in the gel.

A. Purified native C3

B Purified natv C3 and purified properdin mixed and incubated at 37 C for 30 minutes.

C. C3b obtained by trypsination of purified C3

D Aged normal human serum

Arrow denotes application slit. 1) native C3 2) C3b. 3) C3c. Anode is to he right

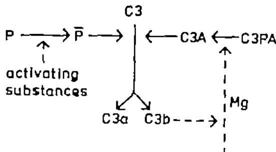


Fig 2 Tentative model of the role of properdin in the alternate pathway

Discussion

The exact mechanism of properdin participation in the alternate pathway of complement activation is not known.

Götze & Müller Eberhard recently postulated a reaction mechanism where active properdin ($\bar{P}$) interacts with native C3 in a way which causes an expression of C3PAse activity C3PAse in presence of Mg^{++} converts C3-proactivator (C3PA) to C3-activator (C3A) which is an enzyme capable of cleaving native C3 to C3a and C3b. C3b initiates the positive C3b-dependent feed-back acting on C3PA together with C3PAse and thus converting C3PA to its active form C3A (*Götze & Müller-Eberhard* 1974)

Our finding suggests that properdin in the activated form acts directly on native C3 converting it to C3a and C3b. W have no evidence that additional factors are required in this reaction.

The evidence that activated properdin directly cleaves native C3 is

1) The C3 and properdin preparations are devoid of C3PA, C3A and C3b, co-factors that hav to be present in the model postulated by *Götze & Müller-Eberhard* (1974)

2) The C3 conversion occurred independently of Mg^{++} and Ca^{++} and was not inhibited by SBTI.

3) Precipitation with IgG-anti-properdin eliminated or diminished the C3 converting property of the properdin preparations.

If any converting enzymes were present in the C3 preparations used, this would result in a C3 conversion when the C3 preparations were incubated in the absence of properdin.

The presence of C3 converting enzymes other than properdin in the properdin preparations is not probable as the fractions containing active properdin were eluated in the slope of the 19S peak on Sephadex G-200 column.

The fact that *Götze & Müller Eberhard* 1 been able to show C3 conversion by purified C3 with purified properdi

to the method used for detecting C3 fragmentation products. By use of small amounts of properdin in these experiments the C3 conversion to C3b is very difficult to reveal with ordinary immunoelectrophoresis. Crossed immunoelectrophoresis is a more sensitive technique.

Our concept of the alternate or properdin pathway of complement activation is shown in Fig. 2.

This work was supported by grants from the *Swedish Medical Research Council* (Grant B75-16X-68-11A) and from the *Medical Faculty University of Lund*.

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AMINOPEPTIDASE ACTIVITY OF *UREAPLASMA UREALYTICUM*

O Vinther and F T Black

Proteolytic activity is a well-known property of some mycoplasma species (1 5 7 8) In particular aminopeptidases have been detected in the membranes of *Acholeplasma laidlawii* (4 10) and *Mycoplasma fermentans* (9) In one case, weak carboxypeptidase activity of *A laidlawii* was also noted (4)

The human T mycoplasmas, being distinguished from other members of the order *Mycoplasmatales* primarily by possessing urease activity have recently been assigned to a separate genus *Ureaplasma* of the family *Mycoplasmataceae* (11) Only one species *Ureaplasma urealyticum* is presently recognized.

In the present study the action of *U urealyticum* cells on a number of synthetic peptidase substrates is reported. By way of comparison, one *Acholeplasma* species and three *Mycoplasma* species were included in the investigation.

Material and Methods

The following organisms were tested for peptidase activity Eight strains representing eight serotypes (3) of *U urealyticum* *M hominis* (PG21) *M fermentans* (PG18); *M pneumoniae* (Mac) and *A laidlawii* (PG8)

U urealyticum was grown in a medium (S) consisting of Tryptic soy broth (Difco) 2.3 per cent w/v horse serum, 16.5 per cent v/v yeast extract corresponding to 1.9 per cent w/v yeast, and sodium penicillin, 1500 i.u./ml. The pH of the final medium was 6.0 The *Mycoplasma* and *Acholeplasma* species were cultivated in a modified Hayflick medium (B) as described previously (6) Determinations of the number of viable organisms in cultures, counted as colony-forming units per ml, were performed on solid S and B media.

Peptides and peptide derivatives used as substrates were Ala_n, n = 2–5 Lys-Ala Ala-Lys; N-acetyl-tri-alanine N-acetyl-tetra-alanine leucine amide hippuryl-arginine, and hippuryl-phenylalanine, all amino acids being of L configuration. The peptides and derivatives were purchased from Sigma Chemical Company St. Louis, Mo., U.S.A.

Organisms to be tested for peptidase activity were grown in 50 ml aliquots of media and harvested at the end of the log growth phase by centrifugation for one hour at 43500 g in a Sorvall RC2b centrifuge. The cell deposits were washed twice in 10 ml phosphate buffered saline twice in 10 ml tris buffer 0.05 M, pH 8.0 and finally resuspended in 50 µl of the latter buffer Uninoculated S and B media, treated in the same way served as references.

Hydrolysis of substrates was assayed in a mixture containing $4–9 \times 10^{8}$ cells of *U urealyticum* or $9 \times 10^{7}–7 \times 10^{9}$ cells of *Mycoplasma* and *Acholeplasma* species, 235 nanomoles peptide or derivative, and 250 nanomoles $CaCl_2$ in a final volume of 70 µl tris buffer 0.05 M, pH 8.0 After incubation for 2 hours at 37 C the mixture was centrifuged to remove the cells and 2 µl of the supernatant was spotted on a thin-layer plate for chromatographic analysis of reaction products.

Precoated cellulose plates (Merck) or silica plates (Gelman Instrument Company) were used. Separations were achieved by developing in the solvent system n-butanol-acetone-acetic acid-water 35 35 10 20 v/v/v/v Leucine and leucine amide were separated as their FLURAM™ derivatives on 5 × 5 cm polyamide sheets (BDH Chemicals Ltd.) by development for 6 min in benzene-acetic acid, 9 1 v/v Fluorescence labelling with FLURAM™ was performed according to the manufacturers suggestions, (Hoffman-LaRoche Inc., New Jersey U.S.A.)

Results and Discussion

The results of peptidase tests are summarized in Table 1

All eight serotypes of *U urealyticum* released alanine from Ala_2. Serotypes VII and VIII were chosen for further experiments and were shown to release free amino acids from all the unsubstituted peptides investigated and from leucine amide. No ninhydrin positive spots ec tected after incubation with N-blo-

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TABLE 1 *Release of Amino Acids from Synthetic Peptidase Substrates by Mycoplasma Species*

Substrates / Organisms	Ala	Ala	Ala Ala,	Lys-Ala, Ala Lys	N Ac Ala,	N Ac-Ala,	LeuNH,	Hippuryl-Phe	Hippuryl-Arg
U ur alyticum	+	+	+	+	0	0	+	0	0
A laidlawii	+	+	–	–	+	+	+	+	+
M pneumoniae	+	+	–	–	0	TR	–	0	0
M hominis	+	+	–	–	0	0	–	0	0
M fermentans	+	+	–	–	0	0	–	0	0

(+)-amino acid released, (0)-amino acid not released, (–) not tested, (TR) trace.

The results clearly indicate that *U urealyticum* possesses aminopeptidase activity whereas carboxypeptidase activity is probably not present. Endopeptidase activity against *N*-acetyl-tri-alanine and N-acetyl-tetra-alanine may also be excluded since such activity would have resulted in release of alanine from liberated Ala_3 or Ala_4. The discovery of aminopeptidase activity in *U urealyticum* adds to the limited knowledge of the biochemical activity of these organisms (2)

It will also be seen from Table 1 that the three *Mycoplasma* species all possess aminopeptidase activity In addition, weak carboxypeptidase or endopeptidase activity may be present in *M pneumoniae*

Both aminopeptidase and carboxypeptidase activities were demonstrated in *A laidlawii* in agreement with results of *Ch les & Gray* (4) *Pecht et al.* (10) on the other hand, were unable to detect release of free amino acids from a number of N-blocked di- and tri-peptides not comprising, however blocked alanine homopeptides.

Studies are in progress in this laboratory to evaluate the possible taxonomic significance of the demonstrated peptidase activities and to further characterize the peptidases in mycoplasma species.

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EOSINOPHIL RESPONSE TO MIGRATING *ASCARIS SUUM* LARVAE IN NORMAL AND CONGENITALLY THYMUS-LESS MICE

Kn d Nielsen Lis Fogh and Sign d Andersen

A marked peripheral and local eosinophilia is a feature of many parasitic infections, e.g. fascioliasis, trichinellosis, lung worm infection and ascariasis. Typically the eosinophil response is elicited during larval migration through the host tissues. Thus, in ascariasis of pigs, peak eosinophil counts are recorded 8–15 days *post infection* (p.i.) i.e. shortly after the larvae have migrated through liver and lungs (*Afon ol & Betts* 1967 *R nens* 1971 *Andersen et al.* 1973) Humoral antibodies appear during the same period (*Taffs* 1964)

Recent studies have given evidence that the capacity to mount an eosinophil response to antigen is mediated by either thymic lymphocytes or lymphoid cells from other sources, i e. spleen, bone marrow or lymph nodes (*Basten & Beeson* 1970 *McGarry et al.* 1971 *Ponzio & Speirs* 1973 1974)

The so-called "nude mouse described by *Flanagan* (1966) is a hairless mutant mouse strain with a rudimentary afunctional thymus (*Pantelouris & Hair* 1970) This mouse was considered convenient as a host model in studies of the eosinophil response to migrating *A suum* larvae.

Thirty-six mice were used. One group consisted of 18 nude mice, while the other group was 18 conventional white mice from a colony that has been maintained as a closed unit at the Instit te for many years. All animals received a primary infection of 1,500 infective *A su m* eggs via a stomach tube Fifty days later, 6 mice of each group received a reinfection dose of 1,500 eggs. Blood samples were drawn from the tail vein daily or on alternate days and a differential white cell count performed on May-Grünwald-Giemsa stained blood films. Mice from either group were sacrificed at daily intervals, starting 4 days p.i. (primary infection) and 10 days p.i. (secondary infection) Specimens for histology were taken from small intestine liver spleen, kidney lungs, heart, lumbar bone marrow and—in the conventional mice—thymus. At sacrifice, blood was obtained by severing the vessels of the neck. Serum was separated by centrifugation and used in indirect haemagglutination test (IHA) using the procedure described by *Herbert* (1967) The antigen used in the test was 1 200 dilution of ultrasonicated *A. suum* uteri with eggs.

Eosin phil counts in the two groups are shown in Fig 1 In the conventional mice there was a distinct rise in eosinophils, peak levels occurring 14–16 days after a primary infection. Following reinfection, the eosinophil response occurred earlier with peak levels 12–14 days p.i., and the counts were higher than after primary infection.

Nude mice showed no eosinophil response after primary infection. After reinfection there was a slight rise at day 12–14 p.i. the significance of which, however is uncertain because the actual number of counts in the reinfected groups were limited and, furthermore, decreasing throughout the experiment due to the daily sacrifices of animals

Serology Conventional mice developed positive IHA-titres 11–12 days after infection or reinfec tion. Titres aried between 1 10 (the lowest dilution employed in the test) and 1:160. Highest titre after primary infection was 1·80 (day 14 p.i.) Prior to reinfection, IHA tests were negative, reaching maximum titres of 1:160 on days 13 and 14 p.i.

In nude mice, the IHA test remained negative, both after primary and secondary infection.

Histology Evidence of larval migration was ascertained in both groups of mice. The lungs contained remnants of migratory tracks, with haemorrhage and cellular infiltrations. However accumulations of mature or immature eosinophils could not be demonstrated in the organs examined, either in the conventional or in the thymus-less mice. The histological ppearance of the bone marrow did not differ from bone marrow histology of noninfected mice with respect to haematopoietic ac tivity

The data presented here confirm that an intact thymus appears to be essential for the cap-

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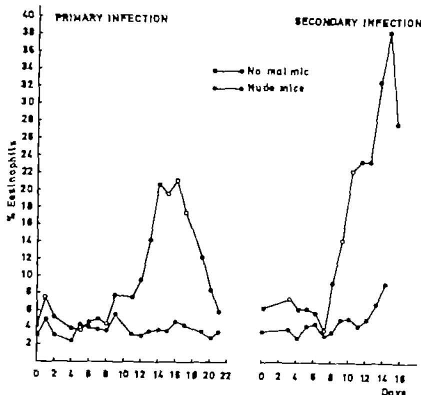


Fig. 1. Average eosinophil counts in *Ascaris suum* infection. Eighteen normal and 18 nude mice received a primary infection. Twelve mice from either group were sacrificed between days 4 and 15 p.i. The remaining 6 mice from either group were reinfected (secondary infection) and sacrificed between days 10 and 15 p.i.

of the animal to mount a peripheral eosinophil response. The results are consistent with those obtained in previous studies of calves with a deficient cellular immune response due to a lack of functional thymic tissue (genetic code A46, *Andresen et al.* 1970). If infected with metacercariae of the common liver fl ke, *Fasciola hepatica*, these calves did not respond with a peripheral eosinophilia whereas normal control calves showed a distinct eosinophil response during larval migration through the liver (*Flagstad et al.* 1972).

The function of the eosinophil granulocyte in immune mechanisms remains unsettled. It is attracted to sites of antigen-antibody reactions and has been proven to phagocytize immune complexes (*Litt* 1963, *Sabesin* 1963). *Speirs* (1963) suggested that eosinophils might be in olved in RNA transfer and thus play a part in antibody synthesis. Evidence of such participation is, however, still lacking.

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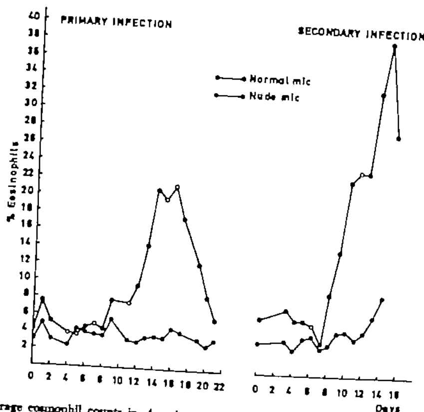


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